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14. ABSTRACT

Breast cancer is the most common cancer among women and the second leading cause of cancer deaths in women today, after lung cancer. Twenty to thirty percent of all breast cancers show overexpressed Her-2/neu, which causes a highly intractable malignant cancer phenotype and almost always spells out poor prognosis and therapeutic outcome. In order to transcriptionally downregulate the Her-2/neu oncogene, we used a combination of a hairpin polyamide and recombinant transcriptional repressor proteins that specifically bind to the AP-2 site on the Her-2/neu gene promoter. Our experiments revealed that the hairpin polyamide HPA-1 was extremely specific to the TATA box of the Her-2/neu promoter and binds with high affinity in vitro, but its in vivo efficacy was limited. However, our experiments with the KRAB and AP-2 based transcriptional repressor proteins showed promise for transcriptional regulation of the Her-2/neu expression. The data from our experiments showed that the recombinant proteins also repress the transcription of other pro-survival genes like cyclin D1 and VEGF. Proliferation studies using WST-1 assays, FACS-cell cycle profile experiments and caspase assays showed that the KRAB-recombinants inhibit cell proliferation and induce caspase3/7 mediated apoptosis. In summary, the KRAB-AP2 recombinant proteins possess the potential to be used as gene therapeutics for Her-2 over-expressing breast cancers and can also be used as gene discovery tools to study cell cycle regulation, cellular proliferation and tumor formation with relation to the genes they regulate.

15. SUBJECT TERMS
Breast cancer, Her-2/neu, Transcription Regulation

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INTRODUCTION

Breast cancer is the most common cancer among women and the second leading cause of cancer deaths in women today, after lung cancer. According to the World Health Organization, more than 1.2 million people will be diagnosed with breast cancer in 2005 year worldwide. The American Cancer Society estimates that this year, approximately 211,240 women in the United States will be diagnosed with invasive breast cancer (Stages I-IV). Approximately 1 in 7 women will develop breast cancer during their lifetime and breast cancer will be responsible for a woman's death is about 1 in 33 (3%). The incidence rate of breast cancer (number of new breast cancers per 100,000 women) increased by approximately 4% during the 1980s but leveled off to 100.6 cases per 100,000 women in the 1990s. The death rates from breast cancer also declined significantly between 1992 and 1996, with the largest decreases among younger women. Medical experts attribute the decline in breast cancer deaths to earlier detection and more effective treatments. However, certain breast cancers in which the Her-2/neu oncogene is overexpressed, have been extremely intractable to therapy and quickly become resistant to most therapies including the novel antibody (herceptin) therapy.

The Her-2/neu gene encodes a 185 Kd transmembrane receptor tyrosine kinase, which has partial homology with other members of the epidermal growth factor receptor (EGFR) family. Her-2 monomers dimerize or oligomerize with themselves or other members of the EGFR family and phosphorylate many downstream molecules, which leads to activation of a variety of signal transduction pathways (1, 2, 3, 4). Some of the well-known pathways are the PI3K/AKT, MAPK, cAMP/PKA (2, 3). Each of these pathways contributes to cell proliferation and cell survival. Oncogenic transformation by Her-2 is caused by disruption of cell cycle regulators (p21 and p27), increased p53 degradation, stimulation of Wnt and NF-kB signaling pathways (reviewed in 5). Her-2 is overexpressed in brain, head & Neck, pancreatic, colorectal, prostrate, ovarian and most significantly in breast cancers (1, 6, 7, 8, 9). Twenty to thirty percent of all breast cancers show overexpressed Her-2, which causes a highly intractable malignant cancer phenotype and almost always spells out poor prognosis and therapeutic outcome (10, 11).

Recent advances in the development of specific Her-2 targeting drugs like the antibody Herceptin have raised hopes for a better prognosis of Her-2 up-regulated cancer phenotype (12). However, development of resistance to Herceptin and other drugs is cause for concern. There is a

great need for developing novel and more effective therapeutics is that target Her-2 at a primordial level, for example- during the transcription of Her-2. Such therapeutics, while showing greater specificity, may also not be susceptible to the development of resistance since a transcriptional repressor of Her-2 would effectively reduce the levels of Her-2 and shut down important survival signaling pathways.

BODY

WORK ACHIEVED FOR THE REPORTING YEAR 2002.

A. Specific aims:

1. Identification of the best binding hairpin polyamide species, which bind with the highest specificity and affinity to the target sequences of the Her-2/neu promoter.
2. Effect of this binding on the normal recognition by promoter binding factors.

B. Studies and results:

Identification of the hairpin polyamides that bind with the highest affinity and specificity to the promoter elements of the Her-2/neu gene.

The most important transcriptional target for down-regulating Her-2/neu gene expression would be the core promoter consisting of the TATA box and the start site of transcription. While designing hairpin polyamides specific for the Her-2/neu TATA box and to minimize non-specific interactions with the TATA box regions of other genes, two nucleotides (-AG-) flanking the 5′ end of the Her-2/neu TATA box were included as part of the recognition sequence for the hairpin polyamides. A promoter database search was carried out to analyze the frequency of this target sequence (-AGTATA-). Sequence analysis of the TATA box regions in the Eukaryotic Promoter Database (EPD) revealed that this -AG- flanking TATA sequence was found in less than 4% of the human type II gene promoters, which include the promoters of c-myc, collagen I, α-fetoprotein, keratin I, opsin, etc.

The hairpin polyamides under study were synthesized at Genesoft Inc., San Francisco, CA by solid phase synthetic methods (2). The hairpin polyamides that could bind to the sequence AGTATA, as determined by the side-by-side pairing rules for these molecules (3), were analyzed for binding specificity using the combinatorial method- Restriction Endonuclease Protection, Selection and Amplification (REPSA) developed in our lab (4). The schematic of REPSA is shown in Figure 1A and 1B. Chemical structures of two hairpin polyamides ImPyPyPy-γ-ImPyPyPy-β-Dp and

ImPyPyPy-γ-PyPyPyPy-β-Dp along with the schematics of their expected consensus DNA recognition sites are shown in Figure 1C. Emergence of preferred binding sequences for the hairpin polyamides in the successive rounds of REPSA is shown in Figure 2A. The binding affinities of the REPSA selected sequences were determined by a Restriction Endonuclease Protection assay (REPA). A representative REPA assay on four REPSA selected sequences for the polyamide ImPyPyPy-γ-PyPyPyPy-β-Dp is shown in Figure 2B. Figure 2C shows the binding affinity (% A) of some REPSA selected sequences determined by REPA for the two hairpin polyamides. DNase I footprinting was carried out to verify exact binding sites in the REPSA selected clones. Dissociation constants were determined using a non-linear regression analysis using a single site binding hyperbolic equation $I=I_{max}$ x [hp]/[k_d=hp], where I is cleavage inhibition, I_{max} is maximum extent of cleavage inhibition observed, [hp] is the hairpin polyamide concentration and k_d is the dissociation constant. Representative footprints for the sequences AGTATA and AGTACA are shown in Figure 3. The k_d values for a few sequences with high affinities are shown in the table below, along with those with a single base pair mis-match (in bold).

binding site	Kd (nM)
ImPyPyPy-γ-PyPyPyPy-β-Dp	
5′-TGTTTT-3′	38
5′-AGTATA-3′	43
5′-TGTTAA-3′	47
5′-**AGTACA**-3′	**216**
ImPyPyPy-γ-ImPyPyPy-β-Dp	
5′-AGTACT-3′	36
5′-AGTACA-3′	54
5′-TGTTCT-3′	42
5′-**AGTATA**-3′	**190**

The data shows that the polyamide ImPyPyPy-γ-PyPyPyPy-β-Dp has high binding affinity for AGTATA along with TGTTTT and TGTTAA but not AGTACA. On the other hand, the polyamide ImPyPyPy-γ-ImPyPyPy-β-Dp has high affinity for AGTACA and very low affinity for AGTATA.

Overall, DNA binding analysis using REPSA and DNase I footprinting showed that the hairpin polyamide ImPyPyPy-γ-PyPyPyPy-β-Dp has a high binding specificity for the sequence –AGTATA-, which fits with our target of the Her-2/neu TATA box. Also, the binding affinity for this sequence is comparable to that of TBP for its consensus TATA sites (5).

N-methyl pyrrole pairings (Py-Py) in a hairpin polyamide recognize either A-T or T-A base pairs in a degenerate manner, but with high affinity. The N-methylimidazole and N-methylpyrrole pairings do not show this degeneracy, that is, Im-Py pairing shows specificity for G-C and Py-Im pairing shows specificity for C-G. However, larger polyamides that recognize G-C rich sequences greater than 6 bp have lower levels of specificity and affinity for those sequences. Due to this reason, we have not yet been able to identify polyamide species that would have a specific interaction with the G-C rich AP-2 binding site of the Her-2/promoter (-GCTGCAGGC-). Smaller polyamides that target a smaller region of the AP-2 site were also not considered because these sites were found to have sequence similarity with the sites for other transcription factors like Sp1. This stringency for sequence requirement is necessary to avoid the potential pitfalls of present day chemotherapeutics. However, with the development of new amide groups which show improved recognition over longer stretches of G-C rich DNA (6, 7), it is possible to generate polyamides that specifically recognize the AP-2 site and other sites that could be important transcriptional targets in the Her-2/neu promoter.

Effect of hairpin polyamide binding on the normal recognition by promoter binding elements.

The TATA binding protein (TBP) as part of the holo-TFIID complex plays a critical role in the transcription of class II genes. TBP forms minor groove contacts with an 8 bp stretch of DNA through its positively charged, saddle-shaped convex surface. Hairpin polyamides also bind in the minor groove of DNA. Hence the molecule ImPyPyPy-γ-PyPyPyPy-β-Dp, which has high affinity for the sequence AGTATA, would have the potential to replace or compete with TBP for binding to the TATA box of Her-2/neu. The ability of this hairpin polyamide to interfere with TBP binding to the TATA box of the Her-2/neu promoter was examined using an electrophoretic mobility shift assay. A 30 bp TBP binding probe consisting of the Her-2/neu promoter DNA from –10 to –39 relative to the transcription start site was chemically synthesized and 3′-labeled with ^{32}P.

This probe (EMTAT-1) was incubated with increasing concentrations of the hairpin polyamide in a buffer containing 10 mM Tris-Cl (pH 7.5), 0.05 mM EDTA, 0.5 mM DTT, 0.01% NP-40, 10 % glycerol and 5 mM $MgCl_2$ for 30 min at 30 ^{0}C. Recombinant human TBP (purified following the procedure described in ref. 8) was added to the DNA and incubated for an additional 30 min. The samples were then electrophoresed on 6 % TBE gels and visualized on a phosphor imager. The polyamide completely inhibits TBP binding at a concentration of 200 nM. This data shown in figure

4A clearly indicates that the hairpin polyamide could effectively inhibit TBP binding to the TATA box.

Reporter assay for preliminary analysis of Her-2/neu down-regulation:

To determine whether inhibition of TBP binding *in vitro* translates into down-regulation of Her-2/neu promoter activity *in vivo*, a luciferase reporter assay was carried out. A Her-2/neu promoter reporter (pNeulit) which contains the promoter sequence from -500 to +30 in a pGL2 vector was transfected into the SKBR-3 breast cancer cells. The cells were treated with the hairpin polyamides ImPyPyPy-γ-PyPyPyPy-β-Dp and ImPyPyPy-γ-ImPyPyPy-β-Dp. The results show that the polyamide ImPyPyPy-γ-PyPyPyPy-β-Dp could inhibit luciferase activity significantly at 200 nM, where as the polyamide ImPyPyPy-γ-ImPyPyPy-β-Dp which has a one base pair difference in binding, could not appreciably do so. This is a clear indication that inhibition of TBP binding to the TATA box may be responsible for down-regulation of Her-2/neu promoter activity by ImPyPyPy-γ-PyPyPyPy-β-Dp. In order to analyze if the AP-2 binding site could be an appropriate target for down-regulating Her-2/neu expression, a pCMV plasmid expressing a recombinant protein, KAP-2 (consisting of a KRAB repressor motif fused to the AP-2 protein), was constructed. The KRAB (krüppel-associated box) motif is a potent repressor of gene transcription, which acts either by histone deacetylase activity and/or co-repressor recruitment (9, 10). This plasmid was co-transfected into the cells along with the pNeulit reporter and luciferase activity was measured after 72 h. The result shown in figure 4B suggests that this repressor protein has a dominant negative effect on the Her-2/neu promoter activity. This not only suggests that the AP-2 binding site is an important target for down-regulating Her-2/neu expression, but also that the KAP-2 repressor may be a promising gene therapeutic with potential for breast cancer gene therapy.

In light of this surprisingly promising result with the KAP-2, we wished to include this repressor protein along with the hairpin polyamides in our original protocol for down-regulating the expression of Her-2/neu oncogene. This would not involve any changes in the original statement of work. An additional gene therapeutic approach would be used in combination with the hairpin polyamides to achieve higher levels of Her-2/neu down-regulation.

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WORK ACHIEVED FOR THE REPORTING YEAR 2003.

A. Specific Aims:

1. In vitro transcription regulation analysis to study Her-2 downregulation by best binding hairpin polyamides and recombinant transcription repressors.
2. Effect of Her-2 downregulation on the proliferation of Her-2 overexpressing breast cancer cells.

B. Studies and Results:

In vitro transcription regulation analysis to study Her-2 downregulation by best binding hairpin polyamides and recombinant transcription repressors.

In our previous report, a luciferase reporter assay was carried out by transiently transfecting the Her-2 overexpressing SkBr-3 cells with the pNeulit (Her-2 promoter reporter) plasmid and treating the cells with the hairpin polyamides or cotransfecting the cells with a recombinant KRAB-AP2 (KAP2) repressor construct. The analysis revealed that both the hairpin polyamide HPA-1 and KAP2 could inhibit transcription from the Her-2 promoter reporter, however the repressor protein showed a three-fold higher level of repression.

In this report, detailed analysis of transcription repression was carried out with the hairpin polyamides and four recombinant constructs of AP-2α, namely AP-2, AP2r, KAP2, KAP2r and AP2rK. AP-2 is the full length AP-2α, AP2r consists of the DNA binding and dimerization domain of AP-2, KAP2r consists of a KRAB repressor domain which was fused N-terminal to the DNA

binding domain of AP-2, KAP2 consists of a KRAB domain fused N-terminal to a full length AP-2 and AP2rK consists of a KRAB domain fused to the C-terminus of the AP-2 DNA binding domain. Domain structures of these fusion constructs are shown in figure 11. These constructs were cloned into the pCMV-tag plasmid (stratagene), which contains a FLAG-tag at the N-terminal of its polycloning site. Expression of these FLAG-tagged recombinant proteins is driven by a CMV promoter.

Different cell lines and reporter constructs were used to study the effects of the hairpin polyamides and the repressor proteins. The cell lines used are the Her-2 overexpressing SKBr-3 and ZR-75-1 and the Her-2 low expressing MCF-7 and MCF-10A. Luciferase reporter assays were carried out on these cells using the reporter plasmid pNeulit, which has the Her-2 promoter consisting of the TATA box and the AP-2 binding site; or an IL-2 promoter reporter containing an optimum (pOPT) or a mutated (pM3T) AP-2 binding site (4). Briefly, the cells cultured in DMEM (containing 10% fetal calf serum) were seeded in six well plates and transiently transfected with 0.4 μg of reporter DNA, 0.1 μg of transfection control DNA (pRLTK) and 0.5 μg effector DNA (pCMV-tag plasmids containing the recombinant DNA constructs) or the indicated concentrations of the hairpin polyamide. After 24 h, the cells were lysed and the luciferase assay was carried out using a dual luciferase assay kit (promega) following the manufacturer's instructions. The data was normalized against the transfection control and plotted.

Increasing concentrations of the hairpin polyamides added to SKBr-3 cells transfected with the pNeulit reporter showed that HPA-1 could inhibit transcription from the pNeulit promoter in a concentration dependent manner, while HPA-2 does not show a significant effect (Figure 5A). This strongly suggests that HPA-1 specifically interacts with the Her-2 promoter and inhibits transcriptional activity. In case of the recombinant proteins, while AP-2α (a known transcriptional activator of Her-2) did not change the reporter luciferase activity compared to a control (empty vector transfection), the three KRAB-repressor proteins showed strong repression, about three fold higher than the repression shown by the highest concentration of the HPA-1 used (Figure 5B). Transient AP-2α expression in SKBr-3 cells does not affect the reporter activity to a significant level because the Neulit expression is already at a very high level in these cells due to various factors that activate the Her-2 promoter.

Increasing concentrations of HPA-1 added to cells transfected with KAP2r showed an additive effect on transcription inhibition from the pNeulit reporter (Figure 6A). The reporter assay with pOPT and pM3T suggests that the transcriptional repression by the KRAB-repressor proteins is specific to the AP-2 binding site containing promoters, with the pM3T reporter not showing a significant change in the cells transfected with the recombinant plasmids (Figure 6B).

To ascertain if HPA-1 and the recombinant fusion proteins indeed downregulate the expression of endogenous Her-2 in overexpressing cancer cells, immuno-cytochemical analysis was carried out in SKBr-3 cell line. The cells were seeded on chamber slides and transfected with the recombinant plasmids or the hairpin polyamide and incubated for 36 h. The cells were fixed in paraformaldehyde, permeabilized with Triton X-100 and immunostained for FLAG tag and endogenous Her-2 with their respective primary and corresponding fluorescent conjugated secondary antibodies. The nuclei were stained with DAPI and the cells were analyzed using a Zeiss Axioplan 2 fluorescence microscope. The results shown in Figure 7 indicate that the cells expressing Flag-tagged AP-2 (stained red with rhodamine) have unchanged Her-2 levels (stained in green with FITC). The KAP2, KAP2r and AP2rK constructs however have a pronounced repressive effect on Her-2 expression, with some cells showing almost no Her-2 staining. The figure also shows clear nuclear localization of AP-2, KAP-2 and KAP-2r, while Her-2 is predominantly membrane localized. The hairpin polyamide however did not induce any changes in Her-2 expression in the SKBr-3 cells. During the next year, Northern and western analysis would be carried out to determine how the recombinant proteins and HPA-1 affect the expression of Her-2 and other important oncogene products and also tumor suppressors like p21 and p27.

Effect of Her-2 downregulation on the proliferation of Her-2 over-expressing cancer cells.

To understand the effect of Her-2 downregulation on the proliferation of breast cancer cells, the cells were seeded in 24 well plates at a density of ~5000 cells per well and transfected with AP-2 or the KRAB-repressor plasmids. The cells were incubated for two days after which they were fed with fresh media and re-transfected with the plasmids. After an additional two days, the cells were washed in PBS and cell proliferation was measured with WST-1 tetrazolium reagent (Roche Biochemicals) following the manufacturer's protocol. WST-1 is a modified MTT reagent with higher sensitivity and linear range compared to MTT. The cells were incubated in the reagent for 30 min and the resulting color was measured at 450 nm. The data was plotted as percentage of cellular proliferation. The results showed that AP-2 slightly decreased the proliferation of the cells, while KAP-2, KAP-2r and AP2rK showed a significant decrease in proliferation of the Her-2 overexpressing SKBr-3 cells and a less significant effect on the Her-2 overexpressing ZR-75-1 cells, but show almost no effect on the proliferation of low Her-2 expressing MCF-7 and MCF10A cells (Figure 8A).

In a similar experiment, SKBR-3 cells were treated with increasing concentrations of HPA-1 and HPA-2 twice over a period of 4 days and cell proliferation was measured, which shows that the hairpin polyamides do not induce a significant proliferation inhibition (Figure 8B). In another

experiment, cells were transfected with AP-2 and KAP2r plasmids and after 6 h, they were treated with increasing concentrations of HPA-1 or HPA-2 and incubated for 48 h. The media was changed and the cells were retreated and re-transfected in the same fashion and further incubated for 48 h. Cell proliferation was measured and the data plotted. The result in figure 8C shows that combination of KAP2r and HPA-1 had a synergistic effect in proliferation inhibition. Over the next year of report, studies involving combinations of HPA-1 and the recombinant proteins with chemotherapeutics like doxorubicin, carboplatin and paclitaxel would be carried out. It should be noted that in all the above proliferation experiments, the data is representative of the total cell population including transfected and untransfected cells. Since the data was not normalized to the number of transfected cells, readers may appreciate this result as comparable to an in vivo situation where all cells in a tumor may not receive the therapeutic treatment.

To further analyze the anticancer activity of the KRAB-repressor proteins in Her-2 overexpressing cancers, a colony formation assay was performed. SKBr-3 cells in six well plates were transfected with 0.5 μg of recombinant plasmids and after three days, the cells were treated with 1 mg/ml of G-418 antibiotic. Under this selective pressure, the cells containing the chromosomally integrated recombinant plasmids would proliferate and form individual colonies. The colonies were stained in crystal violet and photographed. The data in figure 9 shows the cells transfected with the control vector, AP-2 and AP2r could form numerous colonies, while the KRAB fusion proteins showed extremely low colony formation indicating that the KRAB proteins were indeed inhibitory to tumor formation.

In order to understand how the hairpin polyamide HPA-1 and the KRAB proteins decrease the proliferation phenotype of SKBr-3 cells, a TUNEL assay was performed on the cells. The cells were seeded on chamber slides and treated with HPA-1 or transfected with the plasmids. After 4 days, the cells were fixed, permeabilized and TUNEL labeled with fluorescein-12-dUTP. The slides were stained with propidium iodide and mounted. The cells were analyzed under a phase contrast microscope and the data is shown in figure 10. The cells transfected with AP-2 do not show positive TUNEL labeling but about 10-15% of the cells transfected with the KAP2, KAP2r and AP2rK stain positive for TUNEL indicating that at least half of the transfected cells undergo apoptosis, since the transfection efficiency of SkBr-3 in our case was found to be around 20-30% of cells. Similar analysis on the normal breast epithelial cell line MCF10A, which does not overexpress Her-2, revealed that the KRAB fusion constructs do not induce apoptosis in these cells. HPA-1 did not induce apoptosis in either cell line at a concentration of 250 nM. This study suggests that in Her-2 overexpressing cells, Her-2 removal leads to downstream effects, which in turn induce apoptosis.

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WORK ACHIEVED FOR THE REPORTING YEAR 2004.

A. Specific aims for the year of report:

1. Determination of the effect of Her-2 downregulation on the proliferative and physiological response of Her-2 over-expressing cancer cells.
2. Responsiveness of chemo-resistant breast cancers to chemotherapy in presence of the KRAB recombinant proteins.

B. Studies and results:

Determination of the effect of Her-2 downregulation on the proliferative and physiological response of Her-2 over-expressing cancer cells.

We had earlier used the WST-1 assay system (modified MTT assay which has superior sensitivity for cell proliferation studies) to show the effect of the KRAB-recombinants and HPA-1 on cancer cell proliferation (figure 8A and 8B in the 2003 report). We also showed that KAP2 and KAP2r, when used in combination with HPA-1 induce synergistic inhibition Her-2 promoter activity (figure 6A in 2003 annual report) and a strong inhibition of cancer cell proliferation (figure 8C in 2003 annual report).

However, the proliferation assays carried out until now could reveal only partial effects because, in these experiments, only a fraction of the plasmid-transfected cells express the KRAB-recombinant proteins (in transient transfections using Fugene reagent). Hence, in these assays, absolute values of

proliferation inhibition in cells that express the KRAB-recombinant proteins could not be obtained. In order to see the effect of KAP2 and KAP2r expression in only the transfected cells, we carried out a FACS (fluorescence activated cell sorting) analysis. Her-2 over-expressing SkBr-3 and Her-2 normal MCF-7 breast cancer cells were transfected with the plasmids expressing AP2, KAP2 or KAP2r and incubated for a period of 48 h. After the incubation, the cells were dissociated from the surface using a cell dissociation buffer (Life Technologies) and fixed with formaldehyde. The fixed cells were incubated for two hours with a FITC-tagged anti-FLAG antibody which recognizes a FLAG epitope on the recombinant AP-2 and KRAB-AP2 proteins. The cells were washed in phosphate buffered saline three times and then treated with propidium iodide. A cell cycle profile based on propidium iodide staining was generated for the cells. Cell cycle profiles of the FITC positive and FITC negative cells were separated and plotted. The plots of the FITC positive cells would specify the cell cycle profile of a homogeneous population of the transfected cells that express the recombinant proteins. Shown in Figure 12, the different phases of the cell cycle are indicated as M1 (G1 phase), M2 (S phase), M3 (G2/M phase) and M4 (sub-G1 or apoptotic phase). The AP-2 transfected cells show moderate or basal level of apoptosis as observed in untransfected SkBr-3 cells, while the KAP2 and KAP2r transfected cells show high levels of apoptosis, as indicated by an increase in the sub-G1 population of the cells. MCF-7 cells however, do not show appreciable levels apoptosis in the presence of neither AP-2 nor KAP2 and KAP2r (Figure 13).

To substantiate the apoptosis caused by the KRAB-recombinants, a caspase assay was carried out in SkBr-3 cells transfected by these recombinants. In this assay, the cells were transfected with the plasmids expressing the recombinants and split into three sets of triplicates for each transfection. These were plated in 96 well plates and incubated for 40 h. After the incubation, the first set of triplicates was left untreated, the second set was treated with the caspase inhibitor z-VAD-FMK and the third set was treated with the apoptosis inducing ligand, TRAIL. After 8h (total 48 h incubation), the cells were processed using the Apo-One Caspase 3/7 assay system (Promega). In this caspase detection system, the cells are lysed, treated with a rhodamine tagged caspase 3/7 substrate (z-DEVD-R110) and incubated to allow caspase 3/7 cleavage of the substrate, which releases the rhodamine tag. Fluorescence quantification of this released rhodamine using absorption-485nm and emission-530nm wavelengths provides a direct measure of effector caspase activity, which in turn gives an indirect estimation of apoptosis. The data plotted in Figure 14 shows that the control plasmid transfected cells show a basal level of caspase activity after 48 h. The pan-caspase inhibitor z-VAD-FMK inhibited this caspase activity. This caspase inhibitor serves as an additional control to monitor fluorescence increase as authentic caspase activity. The apoptosis inducing ligand, TRAIL, which also serves as a control for monitoring caspase activity, increased caspase activity in the

control cells, indicating apoptosis. Another control, which was transfected with a plasmid expressing only the KRAB domain also shows a similar effect. The AP2 transfected cells showed a small increase in caspase activity, while z-VAD-FMK and TRAIL in these cells showed similar effects as in the control. However the KRAB-recombinant proteins showed a very strong increase in caspase activity, indicating increased apoptosis in their presence. z-VAD-FMK inhibited this caspase activity to a greater extent, while TRAIL showed a high activity in these cells, indicating an overall increase in apoptosis in the cells. The lower levels of caspase activity in the KRAB-recombinant transfected cells compared to the TRAIL treated cells is because of the limitation of transfection efficiency. The recombinants transfect only a fraction of the cells (~20%), while TRAIL essentially enters all cells. The limitation of transfection efficiency is common to all gene based therapeutics. While this limitation would adversely affect therapeutic usefulness, a pragmatic analysis of anticancer efficacy vis-à-vis toxicity should be conducted to evaluate any gene therapeutic. However, with continual advances in gene delivery mechanisms and tumor targeting methods, the therapeutic usefulness of gene-based systems could not be over-emphasized.

I had earlier carried out a colony formation assay to understand the effect of KRAB-recombinant expression on the proliferation of SkBr-3 cells. The data revealed that while cells transfected with AP-2 could form equal number of colonies as compared to cells transfected with a control plasmid in a G418 antibiotic restrictive environment, KAP2 and KAP2r could form only a few colonies (Figure 9 in the 2003 report). Western analysis revealed that the surviving colonies in the KAP2 and KAP2r transfected plates do not express the recombinant protein, strongly suggesting that while AP-2 expression provides a growth advantage to SkBr-3 cells, KAP2 and KAP2r provide a growth disadvantage. The same experiment carried out using MCF-7 cells also shows a similar trend, but not as pronounced as in SkBr-3 cells (Figure 15).

Because the KAP2 and KAP2r constructs confer a growth disadvantage to the cancer cells, I could not generate any stable clones of SkBr-3 cells that constitutively express these recombinant proteins. But in order to perform experiments for understanding the effect of the KRAB-recombinant proteins on cell physiology, one must have a homogeneous population of cells expressing these proteins. To do this, I developed a novel, yet simple FACS based methodology that would rapidly sort out transfected cells as a homogeneous population. In this methodology, the plasmid expressing a recombinant construct is transiently co-transfected into cells along with a GFP (Green Fluorescent Protein) expression vector and the GFP positive cells are selected by FACS. Co-transfection of cells with a 2:1 mix of recombinant plasmid and GFP vector with Fugene reagent has consistently shown that >99% of the FACS selected cells express the recombinant proteins. Up to a million positive cells can be isolated from cultures having low transfection efficiencies. Biochemical analysis (northern

blotting, RT-PCR, western blotting) of these cells after 24 h to 36 h of transfection has excellent advantages compared to stable transfections. These advantages are enumerated below-

1. Due to mostly extra-chromosomal expression, the plasmids do not induce genetic variations in the cells and low levels of GFP expression do not have any noticeable effect on cellular physiology.
2. A short period of high-level expression is preferable for invoking targeted activity rather than a long-term stable expression that could possibly induce generalized effects and alter the genetic and physiological properties of cells.
3. The levels of recombinant protein expression in the cells can be controlled by the amount of plasmid being transfected.
4. Current inducible expression systems show leaky expression in un-induced cells. Leaky expression of AP-2 from un-induced cells has been reported to possess quantifiable transcriptional effects.
5. Virally transduced cells show immunogenic responses, which could interfere with the study of recombinant proteins.

Whole cell lysates were made from these sorted cells and tested for the levels of Her-2 and various other proteins involved in cell cycle regulation and malignant transformation using western blotting. The data shows that cells transfected with KAP2 and KAP2r have lower levels of Her-2, VEGF and Cyclin D1 and higher levels of the cell cycle regulator p27 but not p21 (Figure 16A).

To understand if the changes in the protein levels were caused by transcriptional regulation, RNA was purified from these cells and reverse transcribed into cDNA. A semi-quantitative PCR analysis was carried out using this cDNA. The results of the semi-quantitative PCR analysis show that the mRNA levels of Her-2, VEGF and cyclin D1 are indeed reduced, strongly indicating that the observed reduction in the levels of the respective proteins is due to transcriptional repression (Figure 16B).

Responsiveness of chemo-resistant breast cancers to chemotherapy in presence of the KRAB recombinant proteins.

We carried out WST-1 cell proliferation assays to characterize the effect of the cancer chemotherapeutic drug, doxorubicin, on SkBr-3 cells transfected with the KRAB-recombinant plasmids. Cells were cultured in 24 well plates and transfected with the plasmids in triplicates. After 48 h, 100 nM doxorubicin was added to the cells and the incubation was continued for another 24 h to complete a total incubation period of 72 h. The data shows that doxorubicin induces an additive decrease in proliferation of the cells in presence of all constructs. This suggests that rather than a

strong synergistic effect, the combination treatment of drug and KRAB-recombinant protein shows a weak synergy for proliferation inhibition. However, this effect may be beneficial for cancer therapy since the KRAB-proteins themselves show extremely potent inhibition of cancer cell proliferation. Note that the bar graphs represent proliferation changes in all the cells, while only a fraction of the cells (~20%) are transfected by the plasmid construct. In its present context, the data would be good representation of in vivo combination therapy because present day gene delivery systems would also deliver a therapeutic gene to only a fraction of the cells. However, it is desirable to develop better gene delivery systems for cancer therapy.

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WORK ACHIEVED FOR THE REPORTING YEAR 2005.

A. Specific aims for the year of report:

I had requested a one-year no-cost extension of the project to complete experiments for publication purposes. The experiments are described below.

B. Studies and Results:

Interaction of the KRAB-AP2 recombinants with the AP-2 consensus site on DNA.

Electrophoretic mobility shift assays were perforomed using lysates from the recombinant KRAB-AP2 transfected HepG2 cells. These cells do not express AP-2alpha and provided a background with no interference from endogenous AP2, and hence were suitable for studying the DNA interaction of exogenously expressed KRAB-AP2 proteins. The beta and gamma isoforms of AP-2 did not produce

any cross-reactivity with the oligo used in the study. The HepG2 cells were transiently transfected with the respective recombinant plasmids and nuclear lysates were prepared following the procedure of Schreiber et al (1). The EMSA was carried out using the protocol described previously (2). Briefly, the lysates were incubated with $5'$-^{32}p labeled AP-2 consensus double stranded oligo (Santa Cruz Biotechnology) for 1 h . The samples were resolved by PAGE in 6% TGE gels. The gels were dried and autoradiographed. The data in Figure 18 shows that each AP-2 recombinant is capable of homo-dimerization. The migration of the shifts induced by the KRAB-recombinants in comparison to the shift induced by wild-type AP2, indicate that the DNA interaction is carried out by the homodimers of the respective recombinants. The intensity of the shifted bands suggests that the affinity of consensus site interaction by the recombinants is similar to that of wild-type AP2.

Luciferase assays to determine if the KRAB-AP2 repression of VEGF and Cyclin D1 occurs at specific AP-2 sites on the respective gene promoters.

The TRANSFAC 6.0 algorithm for determining transcription factor binding sites on specific DNA sequences was used to analyze the Cyclin D1 and VEGF promoters for potential AP-2 consensus sites. The analysis revealed several AP-2 sites on each of the gene promoters. These are represented schematically as red boxes in figure 19B and 20B respectively. Promoter constructs of cyclin D1 and VEGF were obtained from Robert Pestell (Department of Oncology, Lombardi Cancer Center, Georgetown University, Washington, D.C.) and Gilles Pages (University de Nice, Nice, France) respectively. The schematics of the promoter constructs shown in figure 19 and 20 indicate the size of each promoter, with respect to the transcription start site. The numbers represent the base-pair length of the promoter from the transcription start site. Each promoter construct was cloned into a luciferase reporter plasmid and these plasmids were co-transfected along with the KRAB-AP2 recombinants in SkBr-3 cells.

The cyclin D1 reporter assay, shown in figure 19A reveals that exogenous AP-2 increases transcription from each of the cyclin D1 promoter constructs containing the AP-2 sites, suggesting that the cyclin D1 promoter does indeed have AP-2 binding sites and that the gene may be potentially regulated by AP-2. The minimal promoter, however did not show any transcriptional activity. This is the first report of AP-2 mediated transcriptional regulation of the cyclin D1 expression. The KRAB-AP2 constructs, expectedly repressed transcription from these constructs. Given the central role of cyclin D1 in cell cycle regulation, we conclude that apart from the repression of Her-2/neu expression, repression of cyclin D1 expression may also be responsible for the strong proliferation inhibition of the KRAB-AP2 constructs.

A similar study using the VEGF reporter, shown in figure 20A revealed that AP-2 upregulates the expression of VEGF, an important growth factor for cellular proliferation and tumor formation. However, in our western blot analysis (Figure 16), AP-2 repressed the VEGF protein levels, as did the KRAB-AP2 proteins. This suggests that AP-2 may play a tumor suppressor role in the context of VEGF expression. However AP-2 is known to upregulate transcription of Her-2/neu, which in turn, upregulates VEGF expression. Hence, AP-2 regulation of VEGF expression in a cellular context may be a complex phenomenon and dependent on several variables. The KRAB-AP2 constructs expectedly repressed the expression of the VEGF reporter more potently than AP-2, suggesting that this effect may also be responsible for the proliferation inhibition in the cells.

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SUMMARY OF KEY ACCOMPLISHMENTS FROM THE RESEARCH PROJECT:

Research accomplishments during the first year (2002) of report:

1. I used a high throughput screen (REPSA) to identify DNA sequences that bind to hairpin polyamides with highest affinity and specificity.
2. I also identified a hairpin polyamide (HPA-1) that shows strong interaction with the TATA box of the Her-2 promoter. Through an EMSA experiment, I showed that HPA-1 could displace the primary transcription apparatus (TBP in the TFIID complex) from binding to the Her-2 core promoter.
3. Luciferase reporter assay using a Her-2 promoter construct showed that HPA-1 indeed down-regulates Her-2 promoter activity, albeit at low levels. Co-transfection of the cells with the recombinant KAP2 repressor protein showed a higher fold down-regulation of the Her-2 promoter. This suggested that the AP-2 binding site is an important target for down-regulating Her-2/neu expression and also that the KAP2 repressor may have potential as a gene therapeutic.

Research accomplishments during the second year (2003) of report:

1. Through luciferase-reporter assays, I showed that HPA-1 and the KRAB-recombinant proteins could inhibit the transcriptional activity of the Her-2 promoter. Immuno-fluorescence studies showed that the endogenous Her-2 expression was dramatically repressed by the KRAB-repressor proteins.
2. The hairpin polyamide HPA-1 by itself was not very effective in inhibiting the proliferation of Her-2 over-expressing breast cancer cells. The KRAB repressor proteins however were more effective in inhibiting the proliferation of the Her-2 over-expressing cells, but not Her-2 low expressing cell lines. Combination of both HPA-1 and the repressor proteins had a strong effect on the proliferation inhibition.
3. The KRAB-recombinant proteins strongly inhibited colony formation in Her-2 over-expressing SKBR-3 cells. In a TUNEL assay, the KAP2 and KAP2r transfected SkBr-3 cells showed apoptotic death. These results strongly suggest that proliferation inhibition by the KRAB repressor proteins could probably be due to the induction of programmed cell death. In the next year of report, detailed apoptosis assays would be carried out in the cells treated with the KRAB constructs and HPA-1 to determine how these agents cause apoptosis. This knowledge could provide impetus for their therapeutic development.

Research accomplishments during the third year (2004) of report:

1. Using FACS analysis, I was able to analyze proliferation inhibition by the KRAB-recombinants in a homogeneous population of the transfected cells. The high percentage of cells in sub-G1 phase of the cell cycle indicates that proliferation inhibition by KAP2 and KAP2r is due to apoptosis induction. This conclusion was substantiated through a caspase 3/7 detection assay, in which increase in caspase activity was monitored in presence of these constructs.
2. I carried out a colony formation assay in MCF-7 cells, similar to that reported earlier in SkBr-3 cells (figure 9, 2003 annual report). The data indicates that the KRAB-recombinants do not induce a sharp reduction in colony formation in the low Her-2 expressing MCF-7 cells. But the cells still seem to possess a growth disadvantage, indicating that apart from Her-2, other programs of gene expression relevant to cancer cell survival may be adversely affected by the KRAB-recombinants.
3. I developed a rapid FACS based transfectant screening protocol for isolating a homogenous population of transfected cells that express the KRAB-recombinants. Using these cells, I carried out western and semi-quantitative RT-PCR analysis for identification of changes in the expression levels of Her-2 and other cell survival and cell cycle regulatory proteins. The

goal was to identify other genes and programs of gene expression that are repressed by the KRAB-recombinants. I have successfully identified that the expression of VEGF and cyclin D1, along with Her-2 are affected by the KRAB-recombinants. This strongly suggests that other survival genes are also repressed by the KAP2 and KAP2r.

4. Treatment of Her-2 over-expressing breast cancer cells with a combination of KRAB-AP2 constructs and doxorubicin did not induce a synergistic effect (where the combined effect of two different therapeutics would have a geometric rather than an additive effect). This however may be an expected result since most of the transfected cells would anyway apoptose. Interpretation of this result as synergistic or additive is complicated by the fact that only a few cells are transfected by the plasmids, while doxorubicin essentially enters all cells.

Research accomplishments during the final year (2005) of report:

1. Electrophoretic mobility shift assays showed that the KRAB-AP2 recombinant proteins bind to the AP-2 consensus sequence with similar affinity as the wild type AP-2.
2. Luciferase reporter assays with cyclin D1 and VEGF promoter constructs, in combination with the western blot data from 2004 year report strongly suggest that the expression of these important genes is regulated by AP-2alpha. The KRAB-AP2 constructs induce a strong transcriptional repression of these genes and hence, are valuable reagents to study cell cycle regulation, cellular proliferation and tumor formation with relation to cyclin D1 and VEGF gene expression.

CONCLUSIONS OF THE RESEARCH PROJECT

The hairpin polyamide HPA-1 is a DNA minor-groove binder with high affinity of interaction with the Her-2 core promoter. This molecule shows potent inhibition of TBP interaction with the core promoter. However, its in vivo transcriptional activity did not compare well against its in vitro DNA binding affinity and lacked therapeutic potential. The Her-2/neu gene promoter contains two AP-2 binding sites and AP-2alpha is known as a major transcriptional activator of Her-2/neu expression. Since, hairpin polyamides suffer from an inherent disadvantage of low cellular uptake, my efforts were instead directed towards the development of recombinant proteins that interact with the AP-2 binding site on the Her-2/neu promoter and repress its transcription. I was able to successfully generate recombinant proteins containing a potent transcriptional repressor domain fused to either the full length or DNA binding domain of the AP2alpha protein. My studies revealed that the KRAB-

AP2 recombinant proteins possessed exceptional affinity of interaction with the Her-2 promoter, due to their inherent helix-span-helix DNA binding domain of AP-2. The KAP2 and KAP2r proteins show potent inhibition of Her-2 promoter activity as was judged from Her-2 promoter reporter studies as well as western blot analysis of endogenous Her-2 protein. Semi-quantitative RT-PCR analysis revealed that these proteins indeed show potent inhibition of Her-2 transcription. They also repress the transcription of other pro-survival genes like cyclin D1 and VEGF.

Unlike AP-2, which seems to induce a growth advantage to Her-2 overexpressing cancer cells, the KRAB-AP2 recombinants seem to induce a growth disadvantage. This is expected since they repress the expression of several survival proteins necessary for cancer cell growth and proliferation. Proliferation studies using WST-1 assays, FACS-cell cycle profile experiments and caspase assays showed that the KRAB-recombinants inhibit cell proliferation and induce caspase3/7 mediated apoptosis.

In summary, the KRAB-AP2 recombinant proteins possess the potential to be used as gene therapeutics for Her-2 over-expressing breast cancers. Concurrently, they can also be used as gene discovery tools and to study cell cycle regulation, cellular proliferation and tumor formation with relation to the genes they regulate. Recent advances in genomic and proteomic analysis as well as systems biological approaches could be used to bring out their complete potential for understanding transcriptional regulation and signal transduction in breast cancer.

PUBLICATIONS.

Y.N. Vashisht Gopal and Michael W. Van Dyke

"A Combinatorial Determination of Sequence Specificity for Nanomolar DNA-binding Hairpin Polyamides".

Biochemistry, 2003 Jun 10; 42(22):6891-903.

REPORTABLE OUTCOMES

Manuscripts in preparation.

Y. N. Vashisht Gopal and Michael W. Van Dyke

"Repression of AP-2 dependent transcriptional programs induces cell cycle arrest and apoptosis in breast cancer cells."

Manuscript under preparation for communication to ***Journal of Biological Chemistry.***

Research projects and Grants.

This work was directly responsible for the development of several research projects in our laboratory, many of which are funded by DOD and other agencies.

Conferences and Workshops Attended.

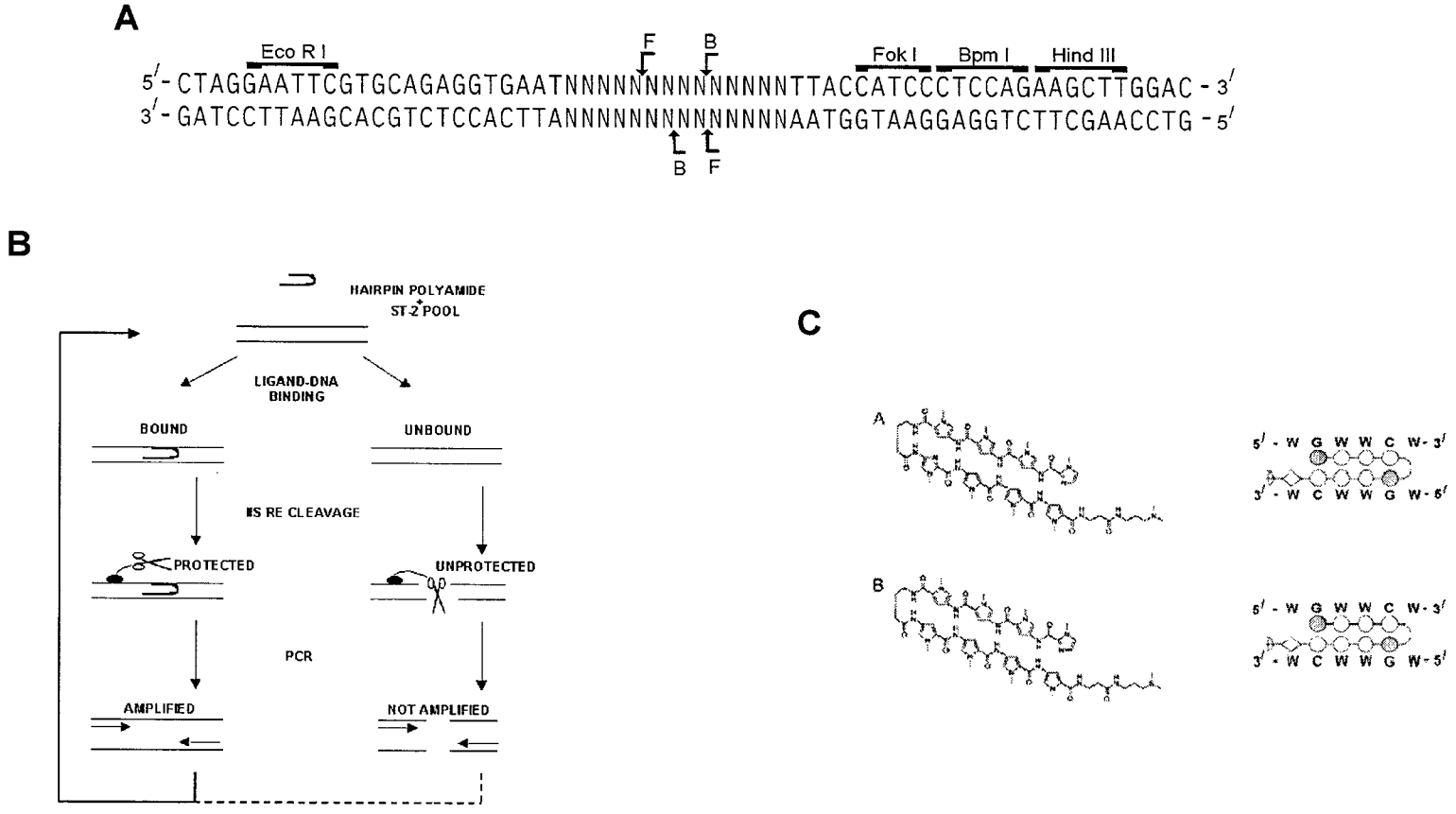
ERA of Hope Breast Cancer Research Program Meeting, Sep 2002, conducted by the U.S. Department of Defense at Orlando, FL.

Cold Spring Harbor Laboratory workshop, "Making and using DNA Microarrays", June 17-24, conducted by the Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Keystone Symposia, NF-κB: Biology and Pathology, January11-16, 2004 at Snowbird, UT.

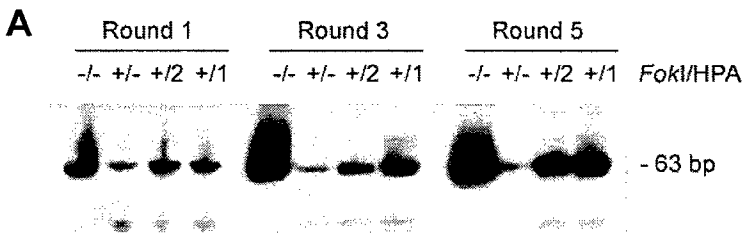
ERA of Hope Breast Cancer Research Program Meeting, June 8-11, 2005, conducted by the U.S. Department of Defense at Philadelphia, PA.

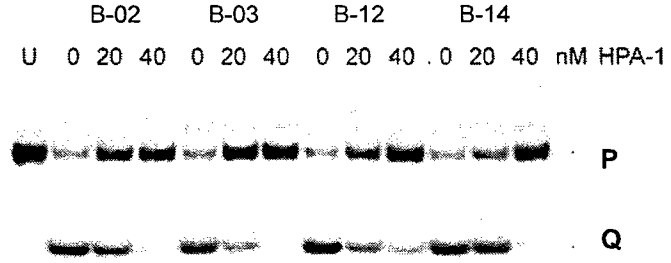
FIGURE 1



(A) REPSA is carried out using a selection template ST2, which is a 63 bp DNA segment, with a 14 base pair random sequence region $(N)_{14}$. The random region is flanked by *Fok I* and *Bpm I* binding sites indicated by brackets over their respective recognition sites. These type IIS restriction enzymes bind to their recognition sites, but cleave within the randon region. The regions within which *Fok I* and *Bpm I* cleave the DNA are represented by F and B. *(B)* Flowchart of the combinatorial method REPSA showing the three steps involved- the ligand binding step, in which the hairpin polyamide is allowed to bind to the ST-2 template; the IISRE cleavage step, in which the enzyme binds sequence-specifically to the template DNA, but cleaves it non sequence-specifically in the random region; and amplification step in which the cleavage protected DNA is amplified and fed into the next round of REPSA to continue the combinatorial selection cycle. The hairpin polyamide is represented by a hairpin structure and the IISRE by an oval bearing a scissors. During each round of the REPSA selection, ligand bound sequences are amplified and unbound sequences are digested. After 5 rounds of selection by *Fok I* and one round of selection by *Bpm I*, highly enriched hairpin polyamide binding sequences arise in the N_{14} region, which are subcloned into pUC19 plasmid and transfected into XL1-blue cells. About 60 clones bearing the enriched templates are selected randomly and the N_{14} region sequenced to determine the hairpin polyamide binding sequences. *(C)* Chemical structures of the hairpin polyamides ImPyPyPy-γ-ImPyPyPy-β-Dp (1) and ImPyPyPy-γ-PyPyPyPy-β-Dp (2) are shown on the left and schematics of their DNA consensus recognition on the right. A pairing of Im (grey circle) with a Py (white circle) recognizes a G·C base pair and vice versa, while a Py-Py pairing recognizes A·T or T·A (complementary A·T and T·A are represented as W·W).

FIGURE 2





C

ImPyPyPy-γ-PyPyPyPy-β-Dp

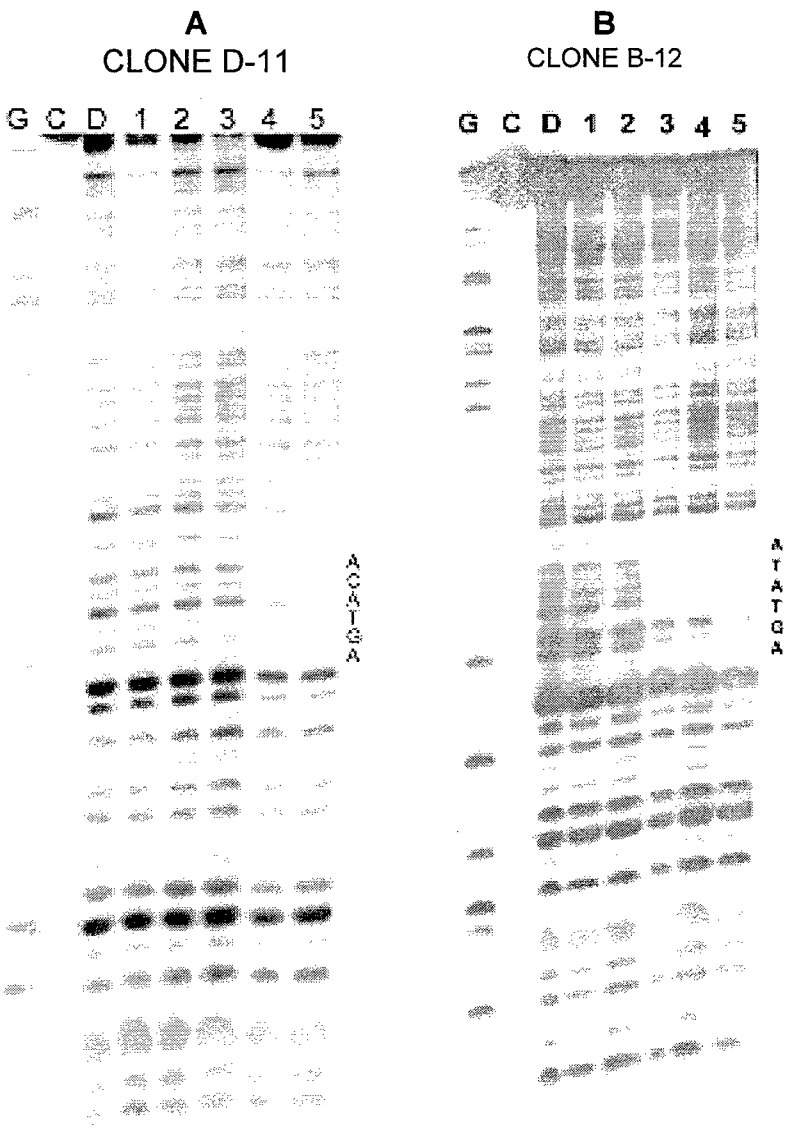
clone	sequence		%A
B-01	ATAGGTACCA**TGTT**	**TT**AC	+++
B-02	CC**TGTTTT**TATAGC	TTAC	+++
B-03	TAAT**TGTTTT**AGTA	TTAC	+++
B-04	**TTGTTTT**TTGCACA	TTAC	+++
B-05	**TGTTTA**TTCTAATT	TTAC	++
B-06	**TAACCTGTTAAAAC**	**TTAC**	**+++**
B-07	GTAT**TGTTAA**CATC	TTAC	+++
B-08	GGCT**TTAACA**TATA	TTAC	+++
B-09	ATAT**TGTTAT**TCAT	TTAC	+++
B-10	**ATGTAAAGTGTTTA**	**TTAC**	**+++**
B-11	**AATTTGATTTTTTT**	**TTAC**	**++**
B-12	TGTTAC**AGTATA**GT	TTAC	+++
B-13	**TCACGATGTAAAGA**	**TTAC**	**+**
B-14	TGT**AGTATA**GGGAA	TTAC	+++
B-15	ATTAC**ATTACT**AGC	TTAC	++
B-16	**GAGTTTATCTTAGT**	**TTAC**	**++**
B-17	CT**TAACT**AGCCTTG	TTAC	++
B-18	ATTTTTTGG**AGATT**	TTAC	+
B-19	**TTTTCT**TGACTATC	TTAC	+
B-20	TAACTAC**AGTTTTT**	TTAC	+
B-21	GT**TCATAA**GCTTGG	TTAC	++
B-22	TGCAATGG**TTTACT**	TTAC	+
B-23	CCTATCC**AGTAAA**T	TTAC	+
B-24	**ATTATCAAAATTGA**	TTAC	++
B-25	TTTATTTT**TAAAGA**	TTAC	+
B-26	TGTCTCGTT**TTATC**	**TT**AC	++
B-27	CCCA**TCTTTA**ACAA	TTAC	+

ImPyPyPy-γ-ImPyPyPy-β-Dp

clone	sequence		%A
D-01	AGTTTCT**AGTTCA**T	TTAC	+++
D-02	AGGTCA**AGTTCA**AT	TTAC	+++
D-03	TAAACTC**AGTTCA**A	TTAC	+++
D-04	TTTTGTAA**ACTTGA**	TTAC	++
D-05	TATTAATTT**AGTTC**	**TT**AC	++
D-06	**TGAGGTAGAAGTTC**	**TTAC**	**++**
D-07	CACTCATT**TGTTCT**	TTAC	+++
D-08	ATTT**TGTTCT**TCAA	TTAC	+++
D-09	**GGTTGAGTACTCAG**	**TTAC**	**+++**
D-10	ACCG**AGTACT**CTGA	TTAC	+++
D-11	GATCTTA**AGTACA**T	TTAC	+++
D-12	TATCCTA**TGTTCA**T	TTAC	++
D-13	AGT**TGTTCA**TTTTA	TTAC	++
D-14	GAAATT**TGTTCA**TT	TTAC	++
D-15	TACAG**TGAACA**ACT	TTAC	+
D-16	CGTCAAT**TGTACT**A	TTAC	+++
D-17	**GTACT**TACACCAAA	TTAC	++
D-18	TTCATT**TGTACT**TT	TTAC	+++
D-19	TTTATCTA**TGTACT**	TTAC	++
D-20	GAACATTGT**TGTAC**	**TT**AC	++
D-21	TGGTCTGGA**TGTAC**	**TT**AC	++
D-22	ATTC**TCAAGT**AACT	TTAC	+
D-23	G**TCTAGT**ATATCTA	TTAC	+
D-24	TGTGC**TGAACT**CAC	TTAC	++
D-25	TGTGC**TGAACT**CAC	TTAC	++
D-26	**TGAACT**TAATCTGT	TTAC	+

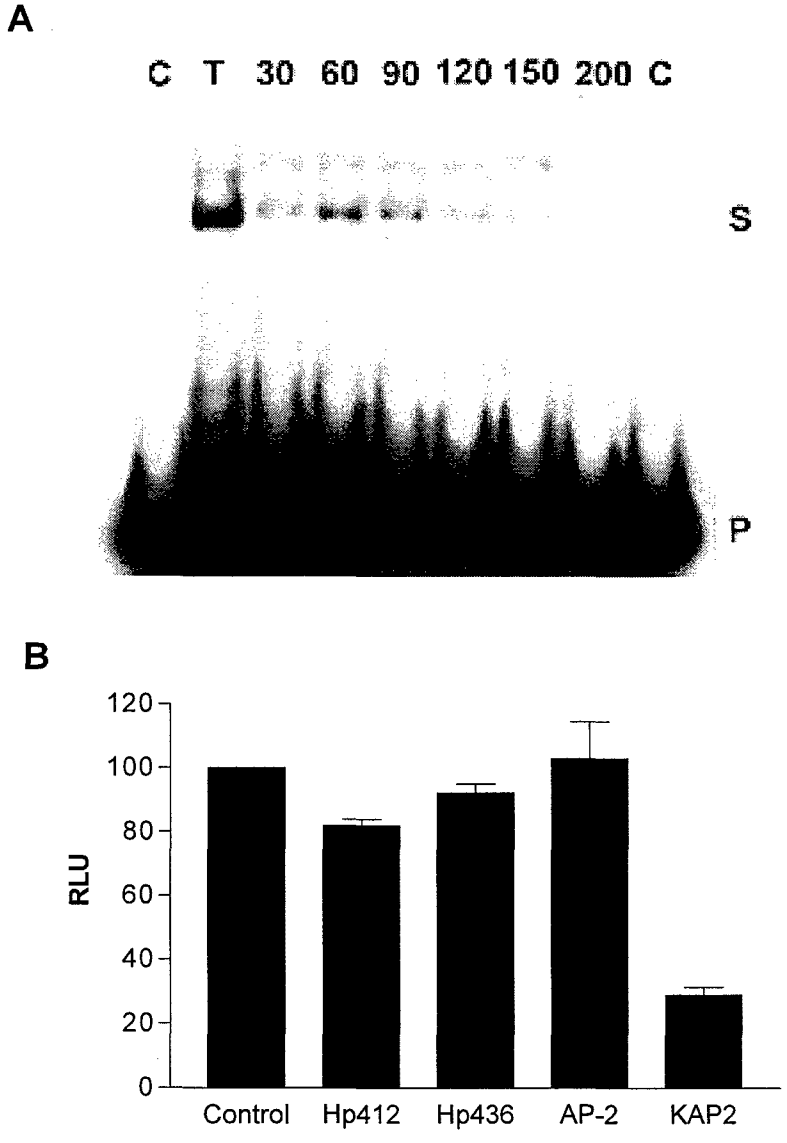
*(**A**)* Emergence of a IISRE cleavage resistant population after five rounds of REPSA. The figure shows ^{32}P-labeled ST2 DNA from the first, third and fifth rounds of REPSA, resolved in a TBE-PAGE gel. ST-2 DNA (**-/-**) was digested with *Fok I* in the absence (**+/-**) or presence of the hairpin polyamides ImPyPyPy-γ-ImPyPyPy-β-Dp (**+/2**) and ImPyPyPy-γ-PyPyPyPy-β-Dp (**+/1**). The results show that in the first round of REPSA, over 95% of the ST-2 DNA was cleaved by *Fok I* in the absence of the hairpin polyamides, while in the presence of the hairpin polyamides, ~ 85% was cleaved. Subsequently in the REPSA selection, DNA incubated with the hairpin polyamides becomes more *Fok I* resistant, as seen in the **+/2** and **+/1** lanes of the third and fifth rounds of REPSA. This *Fok I* resistance indicates the emergence of a significant hairpin polyamide binding population of ST-2 after the fifth round. *(**B**)* Restriction endonuclease protection assay (REPA) to determine *Fok I* cleavage protection conferred by the hairpin polyamide ImPyPyPy-γ-PyPyPyPy-β-Dp on the REPSA-selected clones B-02, B-03, B-12 and B-14. ^{32}P-labeled DNA (**U**) from the REPSA clones was subjected to *Fok I* digestion in the absence (**C**) or presence of 20 nM (**20**) and 40 nM (**40**) ImPyPyPy-γ-PyPyPyPy-β-Dp. '**Q**' indicates *Fok I*-cleaved DNA and '**P**' indicates the DNA protected from cleavage. *(**C**)* REPSA selected sequence clones containing the consensus sites for the hairpin polyamides ImPyPyPy-γ-PyPyPyPy-β-Dp and ImPyPyPy-γ-ImPyPyPy-β-Dp in the 14 base pair random region. Four bases flanking the 3′ end of the random region are also seen. The hairpin polyamide binding consensus sites in the forward or reverse orientation are shown in bold; overlapping sites or a second site are underlined. Clones showing greater than 30%, 50% and 70% cleavage protection at 40 mM hairpin polyamide concentration in the *Fok I* protection assay are indicated by +, ++ and +++ respectively at the right of each sequence (%A).

FIGURE 3



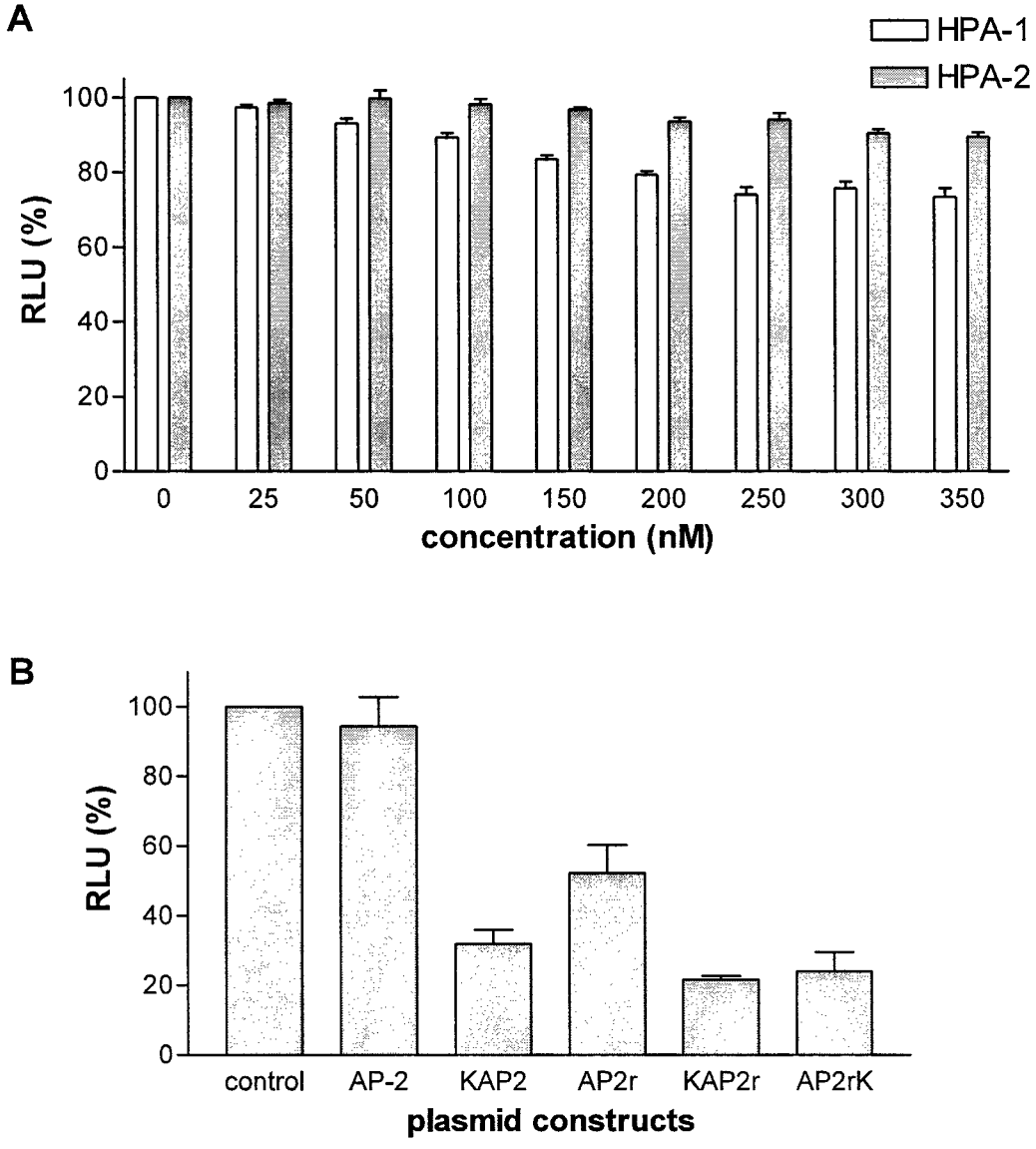
DNase I footprinting for identification of hairpin polyamide binding sites on the REPSA-selected DNA clones D-11 and B-12. ^{32}P-labeled PCR-amplified DNA from the clones (lane **C**) was incubated with 10, 20, 40, 60 and 80 nM (lanes **1**, **2**, **3**, **4**, **5**) of ImPyPyPy-γ-ImPyPyPy-β-Dp ***(A)*** and ImPyPyPy-γ-PyPyPyPy-β-Dp ***(B)*** for 1 h. at 37 ^{0}C. The DNA was then subjected to DNase I cleavage (lane **D**) at RT for 30 s. Products of G-specific chemical sequencing reaction (lane **G**) served as electrophoretic markers. The DNase I digestion products were resolved on a high resolution denaturing PAGE and visualized by autoradiography. The regions of the footprints corresponding to the hairpin polyamide binding sites (AGTACA and AGTATA) are represented at the right of the footprint.

FIGURE 4



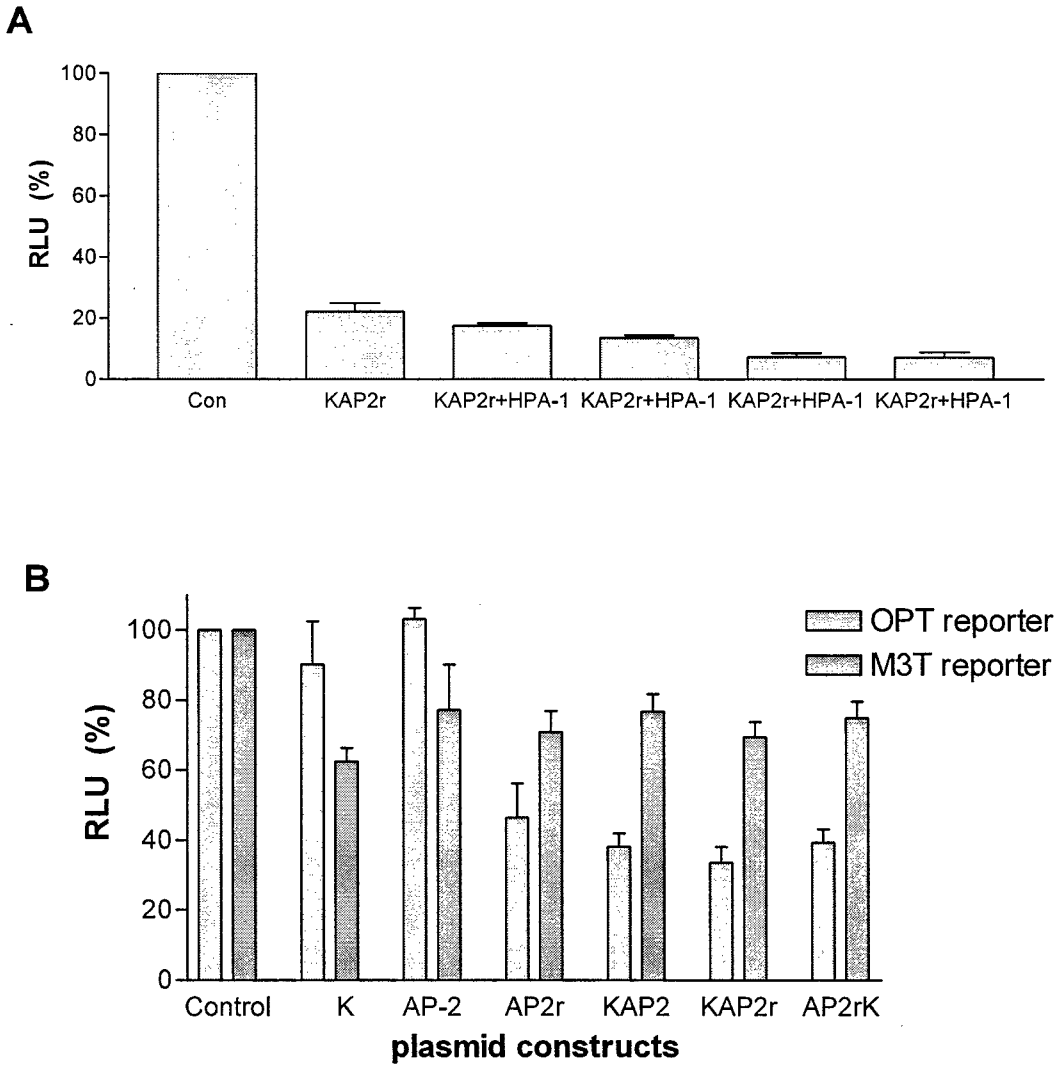
(A) Electrophoretic mobility shift assay on the EMTAT-1 probe (lane C) showing the shift induced in the DNA by TBP (lane T). Increasing concentrations of the hairpin polyamide ImPyPyPy-γ-PyPyPyPy-β-Dp in nM concentration are indicated over the respective lanes. **P** and **S** on the right of the gel indicate the free probe and the TBP shifted probe. The hairpin polyamide was able to completely inhibit TBP binding at a concentration of 200 nM. **(B)** A Luciferase reporter assay carried out in SkBr-3 cells. A Her-2/neu promoter reporter plasmid (pNeulit) was transfected at a concentration of 1μg into 50,000 cells using the Fugene 6 reagent. After 6 h. incubation, the hairpin polyamides (200 nM) were added and the cells incubated for 72 h. Similarly, 1μg of eukaryotic expression plasmids expressing AP-2 (pCMV-AP2) and KAP-2 (pCMV-KAP2) were co-transfected into the cells along with the reporter and the cells were incubated for 72 h. A β-galactosidase control vector (pSV-βgal) was used as a transfection control. After incubation, the cells were lysed and the luciferase activity was measured using a promega luciferase assay system. The data was expressed as relative light units in a mean of triplicates histogram. On the x-axis, Control shows the luciferase activity in cells transfected with pCMV empty vector, AP-2 and KAP-2 show the luciferase activities in cells transfected with pCMV-AP2 and pCMV-KAP2. Hp436 and Hp412 show the luciferase activity in cells treated with the polyamides ImPyPyPy-γ-PyPyPyPy-β-Dp and ImPyPyPy-γ-ImPyPyPy-β-Dp.

Figure 5



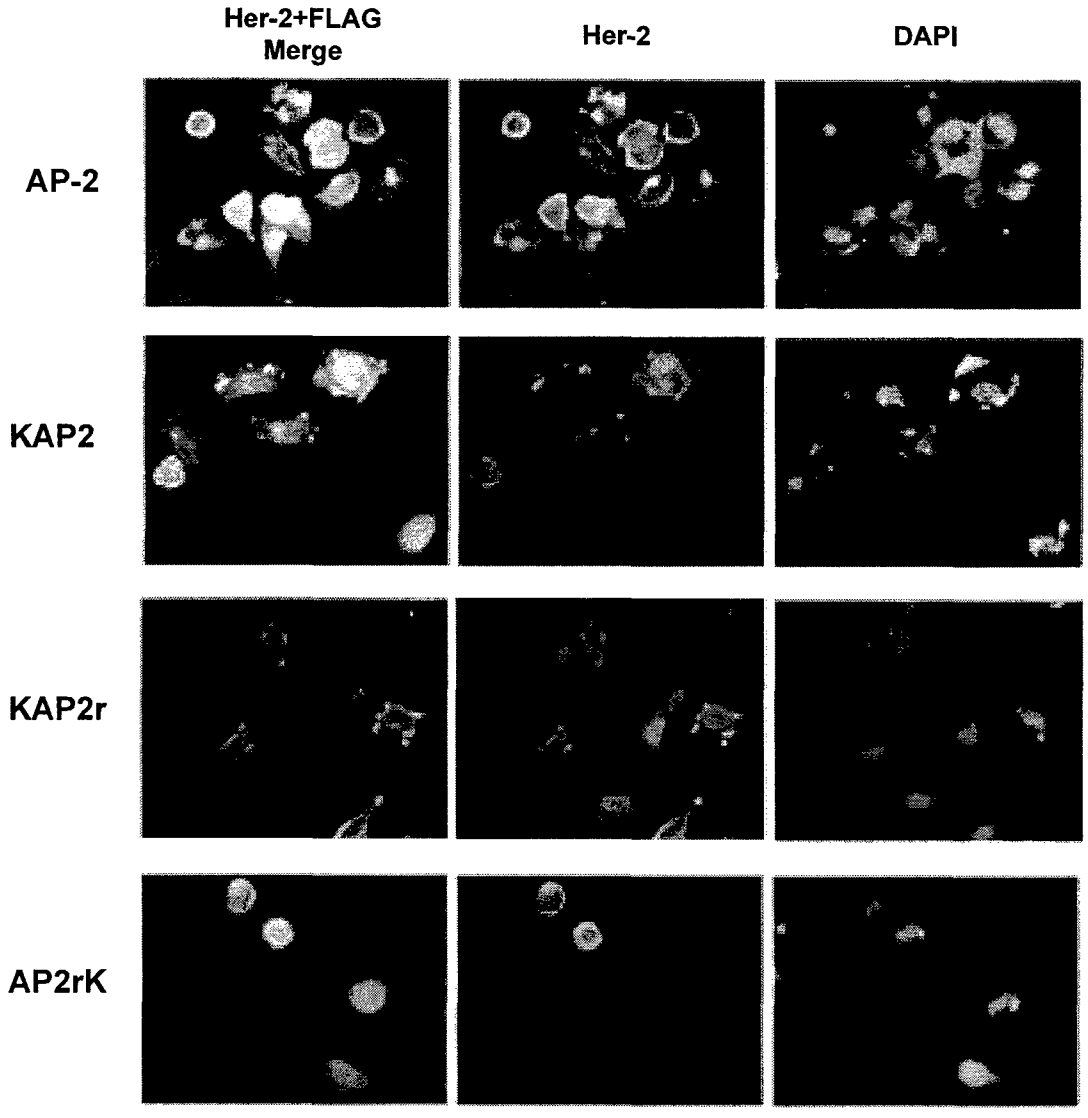
(A) SkBr-3 cells were seeded on 6 well plates at a concentration of 10^5 cells per well and transfected with 0.4 μg of pNeuLit plasmid, 0.1 μg of pRLTK plasmid and 0.5 μg of empty vector (pCMVtag) using fugene 6 reagent. After six hours, the cells were treated with increasing concentrations of the hairpin polyamides HPA-1 or HPA-2. The cells were incubated for an additional 24 h, lysed and luciferase activity was measured in the lysates. Firefly luciferase activity was normalized against renilla luciferase activity and the data was plotted as percentage of Relative luciferase activity (% RLU). The data shows that the Her-2 promoter activity is progressively inhibited by increasing concentrations of HPA-1, but not by HPA-2. The highest level of inhibition was observed at a concentration of 250 nM HPA-1, after which there was no significant change. **(B)** SkBr-3 cells were seeded in 6 well plates and transfected with 0.4 μg of reporter plasmid (pNeuLit), 0.5 μg of the effector plasmid (AP2, AP2r, KAP2, KAP2r or AP2rK) and 1 μg of transfection control plasmid (pRLTK) with fugene 6 reagent. The cells were incubated for 24 h after which the cells were lysed and the luciferase activity measured. As described above, the data was normalized and plotted. The plot shows that while AP-2 does not change the luciferase activity significantly, the KRAB-repressor proteins have a strong repressive effect.

Figure 6



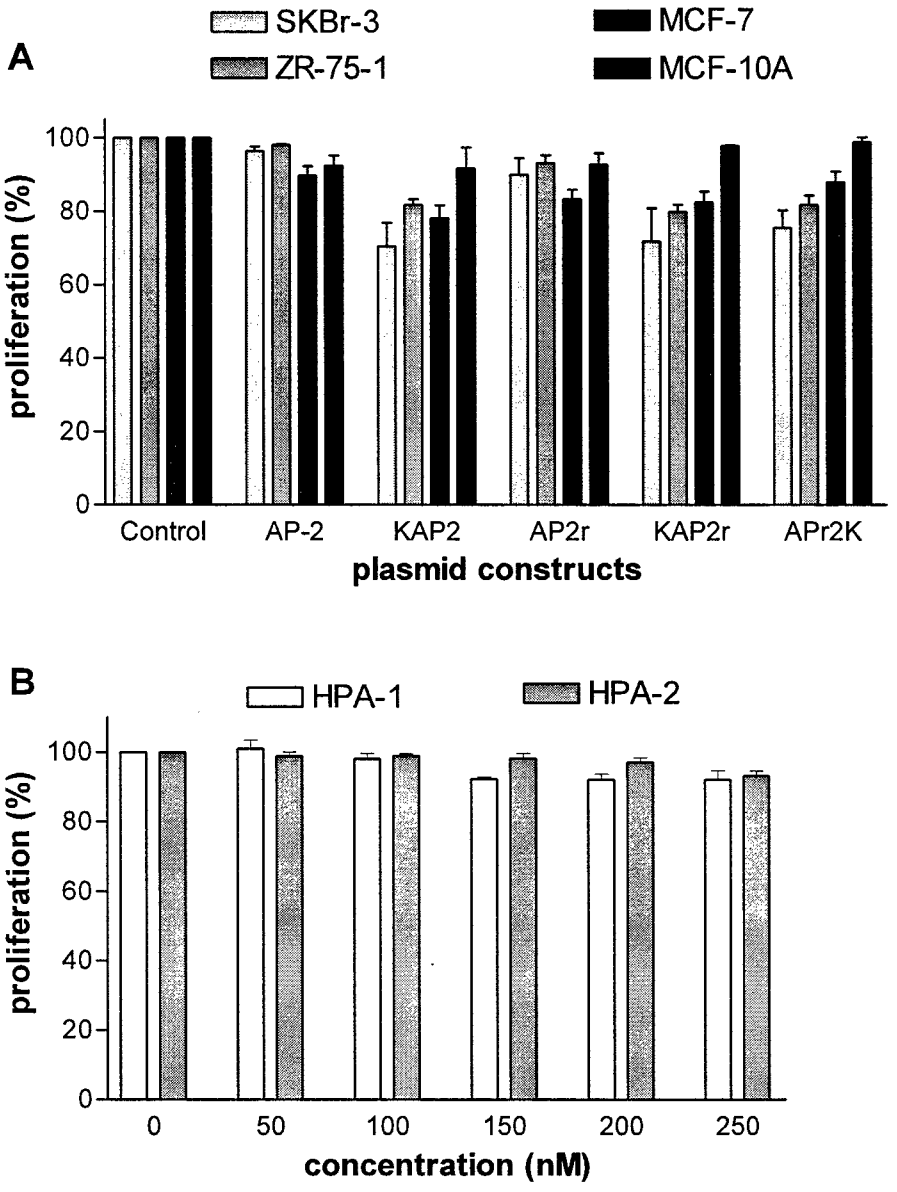
(A) SkBr-3 cells in six well plates were transfected with pNeulLit, KAP2r and pRLTK and six hours after transfection, increasing concentrations of HPA-1 was added to the cells. The cells were lysed, luciferase activity was measured and the normalized data was plotted. The plot shows that the combination of KAPr2 and HPA-1 had an additive effect on the repression of Her-2 promoter activity. **(B)** AP-2 reporter assay was carried out in SKBr-3 cells using pOPT (promoter with optimal AP-2 site) or pM3T (promoter with mutated AP-2 site) plasmids under the same conditions as described above. The data shows that while AP-2 induces a slight increase in the pOPT reporter activity, the KRAB repressors induce a strong repression. Such changes in the reporter activity were not observed in lysates from cells transfected with the M3T reporter.

Figure 7



SkBr-3 cells seeded in chamber slides were transiently transfected with 0.3 μg of AP2, KAP2, KAP2r and AP2rK expression plasmids and were subjected to immuno-fluorescence labeling after 36 h. Cells were probed with a FITC-labeled 2^{O} antibody for Her-2/neu and a rhodamine labeled 2^{O} antibody for a FLAG tag expressed as an N-terminal peptide tag in all the plasmid constructs. Untransfected SKBr-3 cells show high expression of Her-2/neu, which is predominantly membrane localized (green). Transfected cells show predominant nuclear localization of the exogenously expressed AP-2 and KRAB-repressor proteins (red flag-tag). While expression of AP-2 slightly increases the membrane bound Her-2/neu, expression of KAP-2, KAP-2r and AP2rK strongly represses Her-2/neu levels, with some cells showing complete absence of Her-2/neu. Similar results were obtained with 24 h incubation after transfection. This data strongly indicates that the recombinant KRAB-repressor proteins not only express active proteins that nuclear localize, but also bind specifically to the AP-2 binding element in the Her-2/neu gene promoter and downregulate transcription from this gene.

Figure 8 (A & B)



(A) SKBr-3, MCF10A, ZR-75-1 and MCF-7 cells were seeded in 24 well plates at a density of $5x10^3$ cells per well and were transfected twice with 0.3 μg of AP-2 and the KRAB-repressor plasmids over a period of 4 days (first transfection at 0 h and second transfection at 48 h). The cells were then washed with PBS, treated with WST-1 tetrazolium reagent, incubated for 30 min and the change in the color of the media was recorded using a plate reader at 450 nm. The data was plotted as percentage proliferation, which showed that AP-2 induced a small decrease in proliferation and the KRAB-repressor proteins caused significant proliferation inhibition in SkBr-3 and ZR-75-1 cells while no significant change was noticed in the Her-2 low expressing MCF-7 and MCF-10A cells.
(B) SKBr-3 cells were treated with 50, 100, 150, 200 and 250 nM of HPA1 or HPA-2 and proliferation twice over a 4-day period (as above) and proliferation was measured with WST-1 reagent. The results show that both drugs do not show a significant proliferation inhibition.

Figure 8 (C)

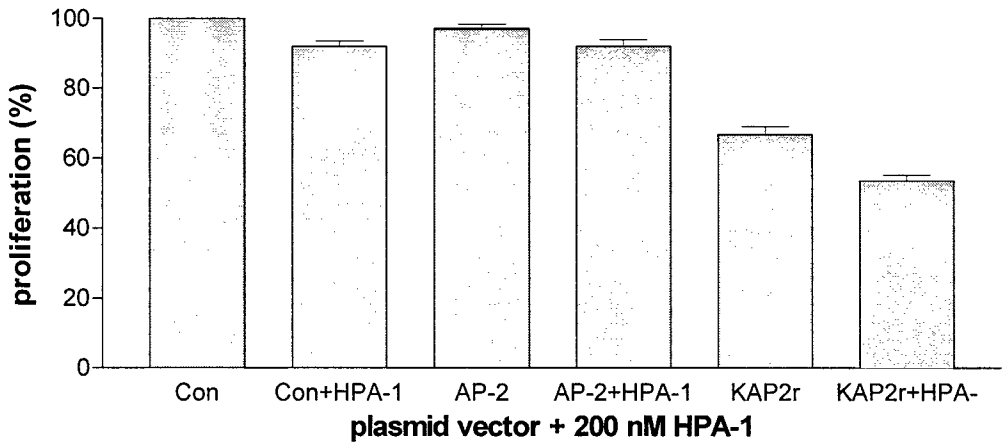


Figure 8: **(C)** SKBr-3 cells were transfected with AP-2 and KAP2r plasmids; after 6 h, they were treated with 250 nM of HPA-1 or HPA-2 and incubated for 48 h. This was followed by a second transfection followed by a second drug treatment. Cell proliferation was measured using the WST-1 tetrazolium reagent. While HPA-1 and HPA-2 do not influence cell proliferation by themselves, combination with KAP2r sensitizes the SKBr-3 cells to HPA-1 and this causes a slight increase in proliferation inhibition. The same is not true for cells transfected with AP-2. This may indicate a synergistic effect of the KAP2r construct and HPA-1 on the proliferation of Her-2/neu overexpressing SKBr-3 cells.

Figure 9

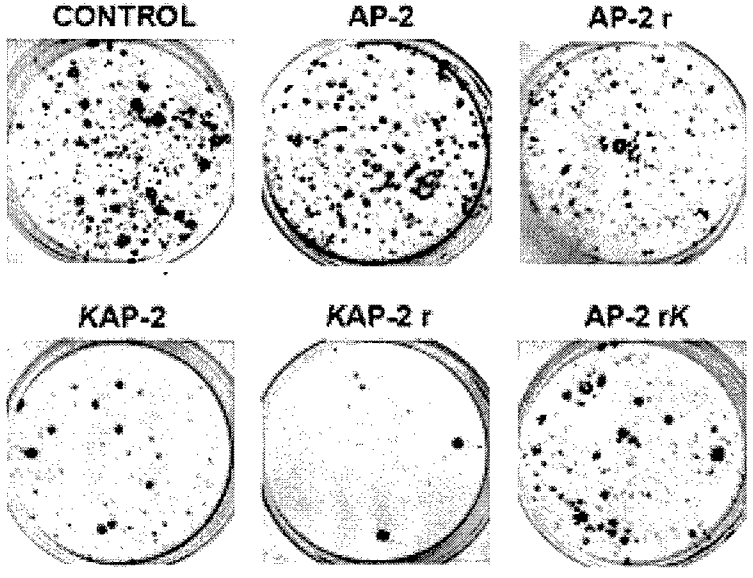
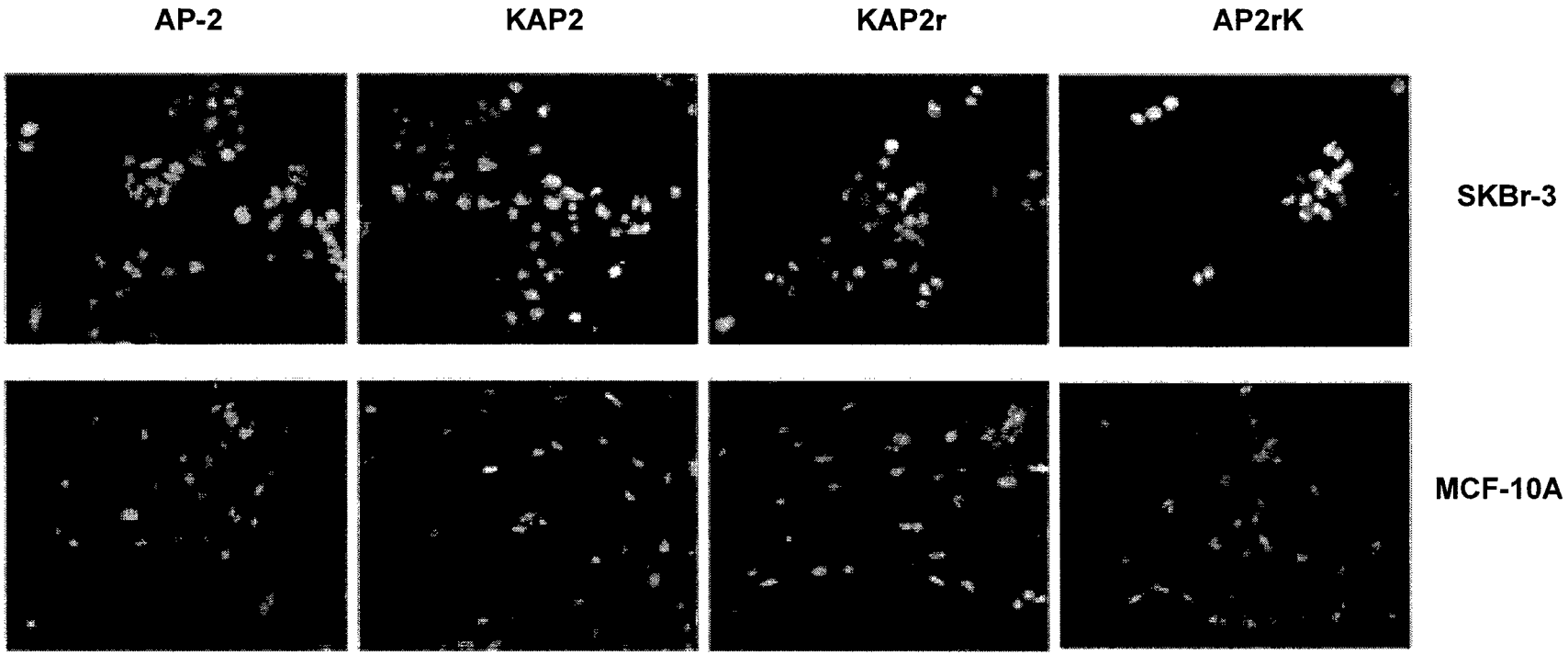


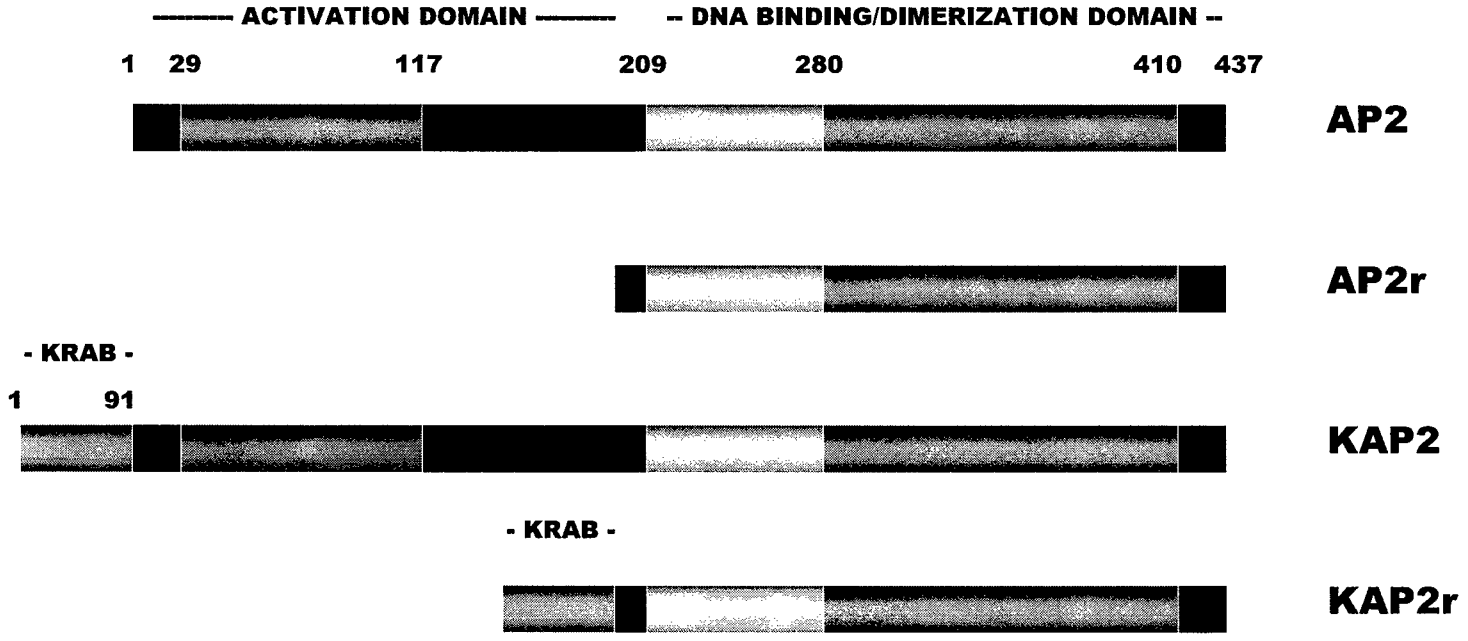
Figure 9: SkBr-3 cells in 6 well plates were transfected with 0.5 μg/well of the recombinant plasmids. A *Neo*^{r} gene in the plasmid allows for G-418 antibiotic selection of cells that stably integrate the plasmid. Stable cell selection was carried out in presence of 1 mg/ml of G-418 over a period of 4 weeks. G-418 resistant colonies were then stained with Crystal violet. The data showed that while stable AP-2α expression did not affect colony formation compared to the control plasmid, AP-2r showed a small reduction in the number of colonies. The KRAB-repressor proteins however significantly inhibited colony formation.

Figure 10



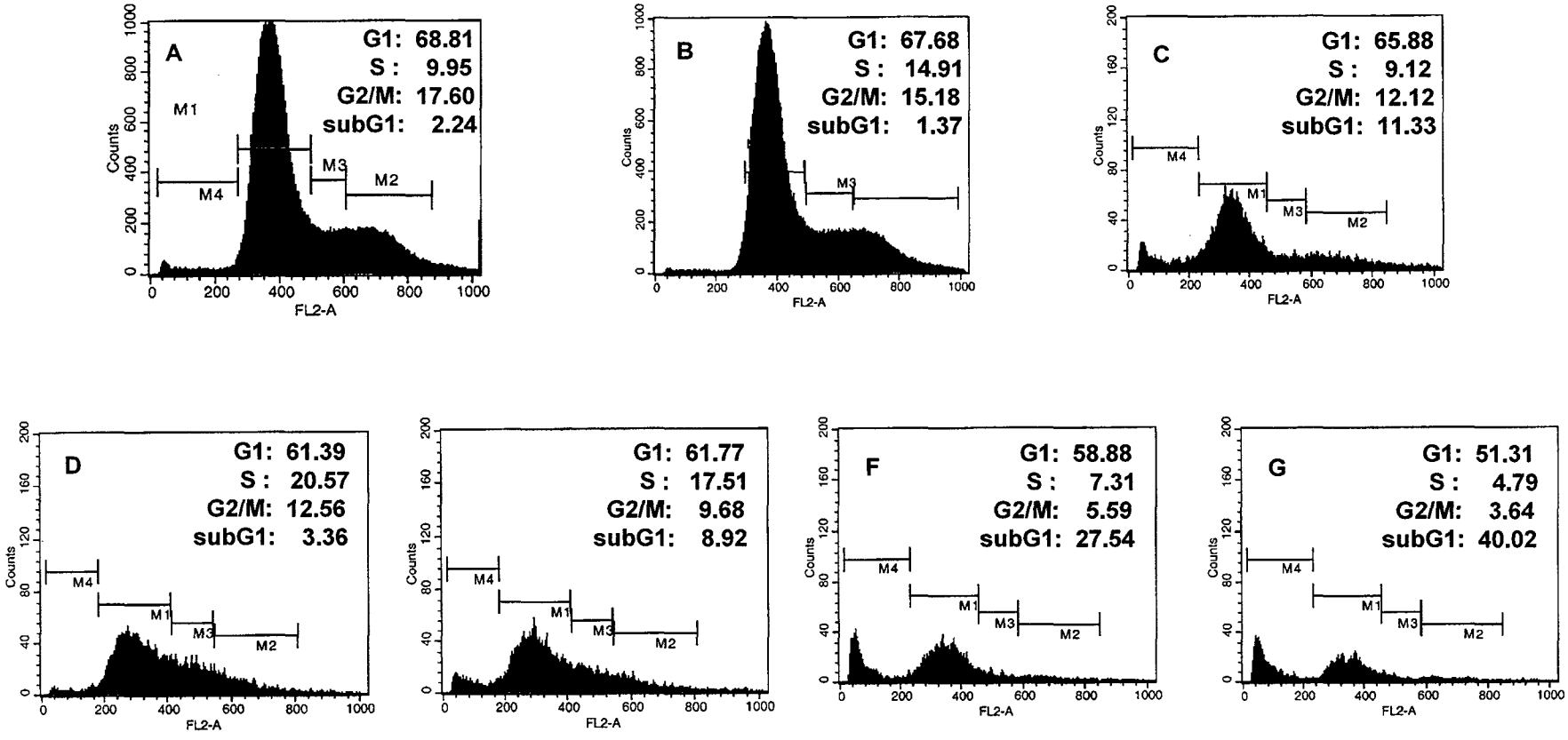
SkBr-3 and MCF-10A cells were transfected with the plasmids and after 4 days of incubation, TUNEL labeling with fluorescein 12-dUTP was carried out. The cells were then propidium iodide stained, visualized by fluorescence microscopy and the green and red images were superimposed. Transient AP-2 expression does not induce apoptosis in SkBr-3 cells, while KAP2, KAP2r and AP2rK expression induces apoptosis in about 10-15% of the cells. However, no apoptosis was observed in the MCF-10A cells transfected with either AP-2 or the KRAB-repressor constructs. This directly correlates with the anti-proliferation effect of the KRAB-repressor proteins, strongly suggesting that the anti-proliferation activity of the KRAB-repressor proteins in SkBr-3 cells is at least in part a result of apoptosis induction.

Figure 11



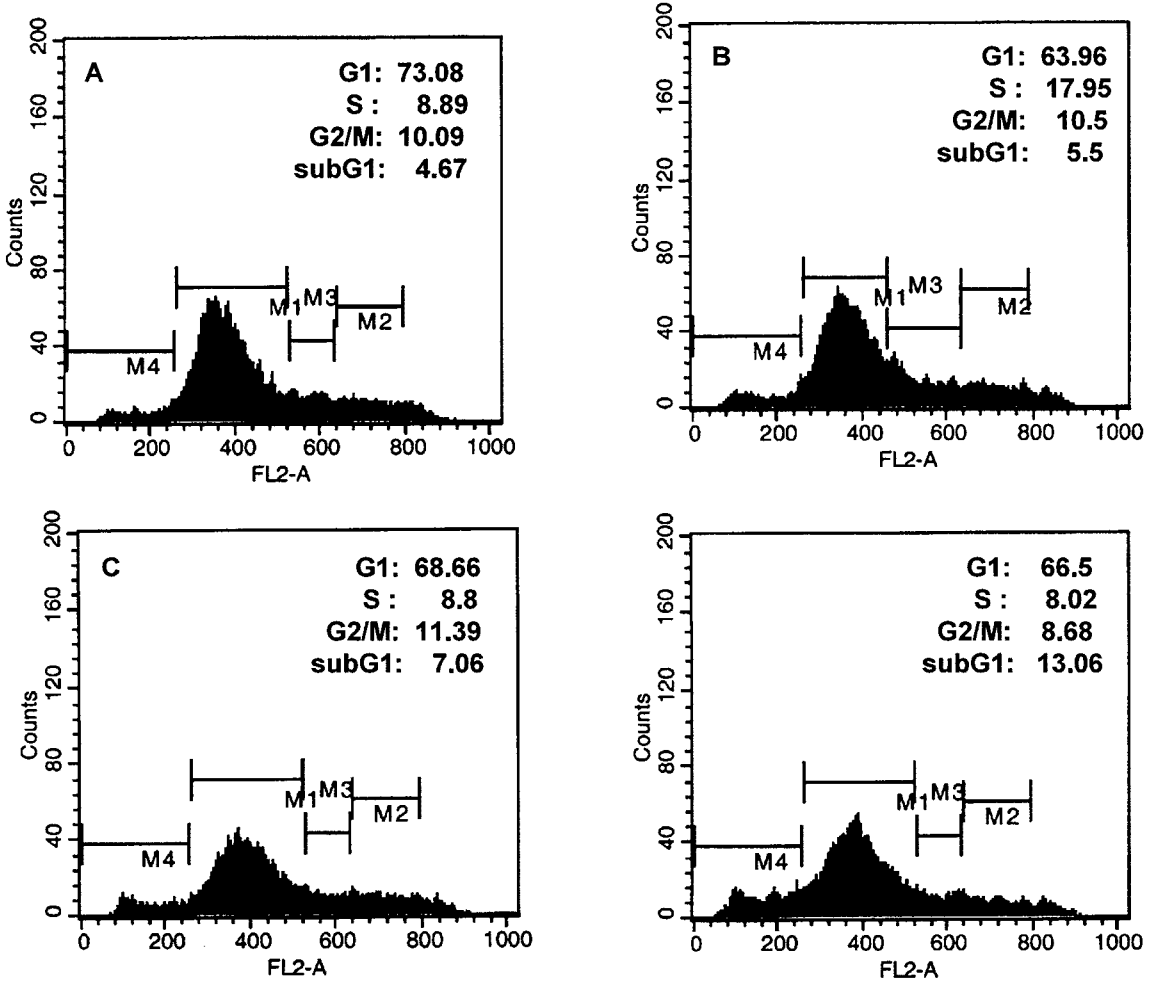
Shown above are the domain structures of the different AP-2 recombinant constructs cloned into a pCMV-tag vector. **AP2** is the human AP2alpha protein is a 437 amino acid, 52 Kda protein with a distinct activation domain and DNA binding/dimerization domain. The domain locations are indicated by the number of amino acids spanning each domain. The construct **AP2r** lacks the activation domain, but retains the DNA binding and dimerization properties. **KAP2** is the full length AP2alpha with a KRAB domain fused to the N-terminus of the protein. **KAP2r** is AP2r with a KRAB domain fused to the DNA binding/dimerization domain. Not shown above is another construct that expresses only the KRAB domain (named as **K**). Our studies have consistently shown that when **K** is expressed in cells, it localizes in the cytoplasm and has no apparent effect on cell physiology. Hence the **K** plasmid served as a good negative control for our experiments. An immunogenic FLAG-tag on the plasmids helps in easy immunogenic recognition of these proteins. The vector contains a G418 resistance marker to carry out colony formation assays and stable clone selection in G-418 antibiotic containing growth medium.

Figure 12



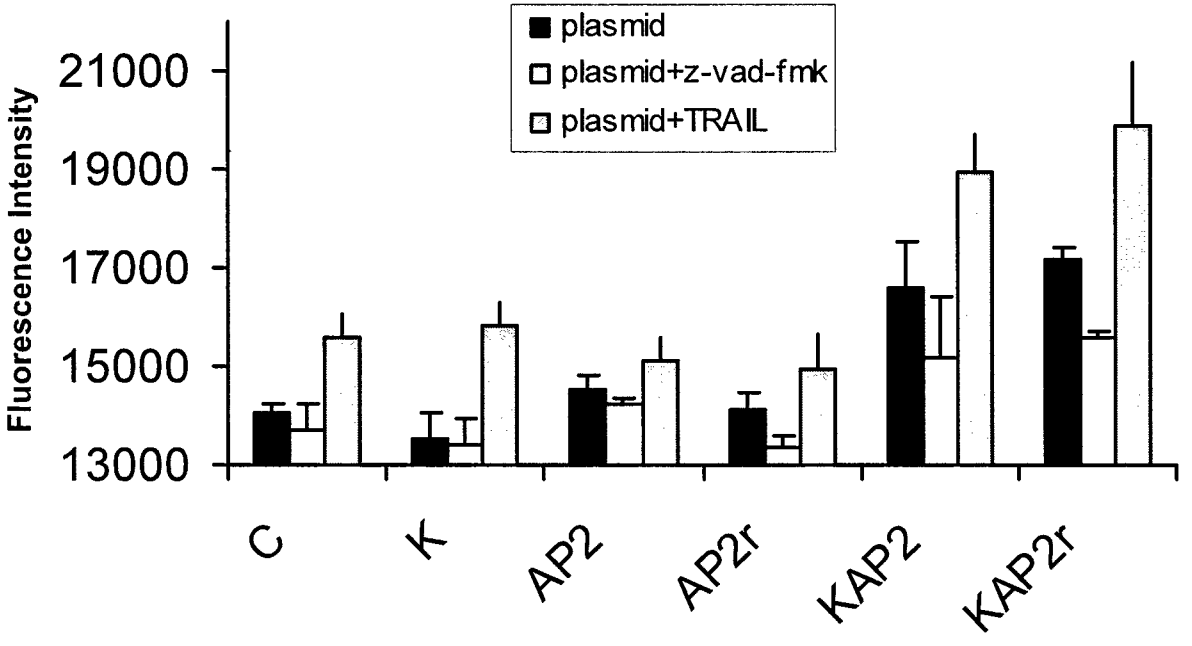
Transiently transfected SkBr-3 cells were incubated for 48 h, fixed and stained with FITC-anti Flag antibody for two hours. The cells were then stained with propidium iodide and a cell cycle profie was generated using flow cytometry. Figure A shows the profile of untransfected and transfected cells from AP2r transfected cells. Figure B shows the profile of untransfected cells alone. Figure C shows the profile of the AP2r transfected cells alone. Figure D, E, F and G are profiles of cells transfected with K, AP-2, KAP2 and KAP2r respectively.

Figure 13



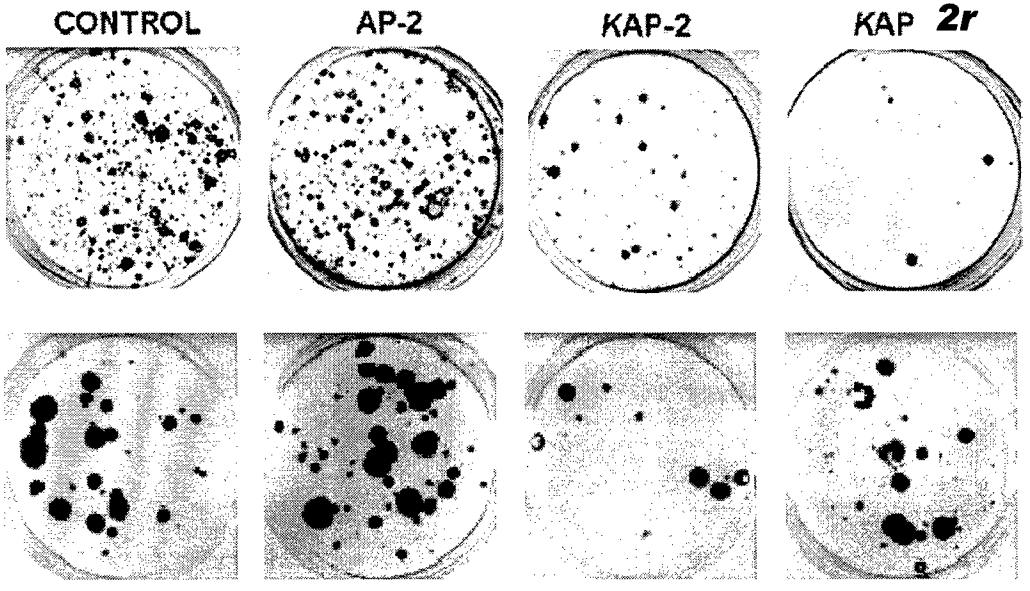
Cell cycle profile of K (**A**), AP2 (**B**), KAP2 (**C**) and KAP2r (**D**) transfected MCF-7 cells show lower numbers of cells in the sub-G1 phase of cell cycle compared to SkBr-3 cells. This suggests that the KRAB-recombinants readily induce apoptosis in high Her-2 expressing cells, while inducing lower levels of apoptosis in the low Her-2 MCF-7 cells.

Figure 14



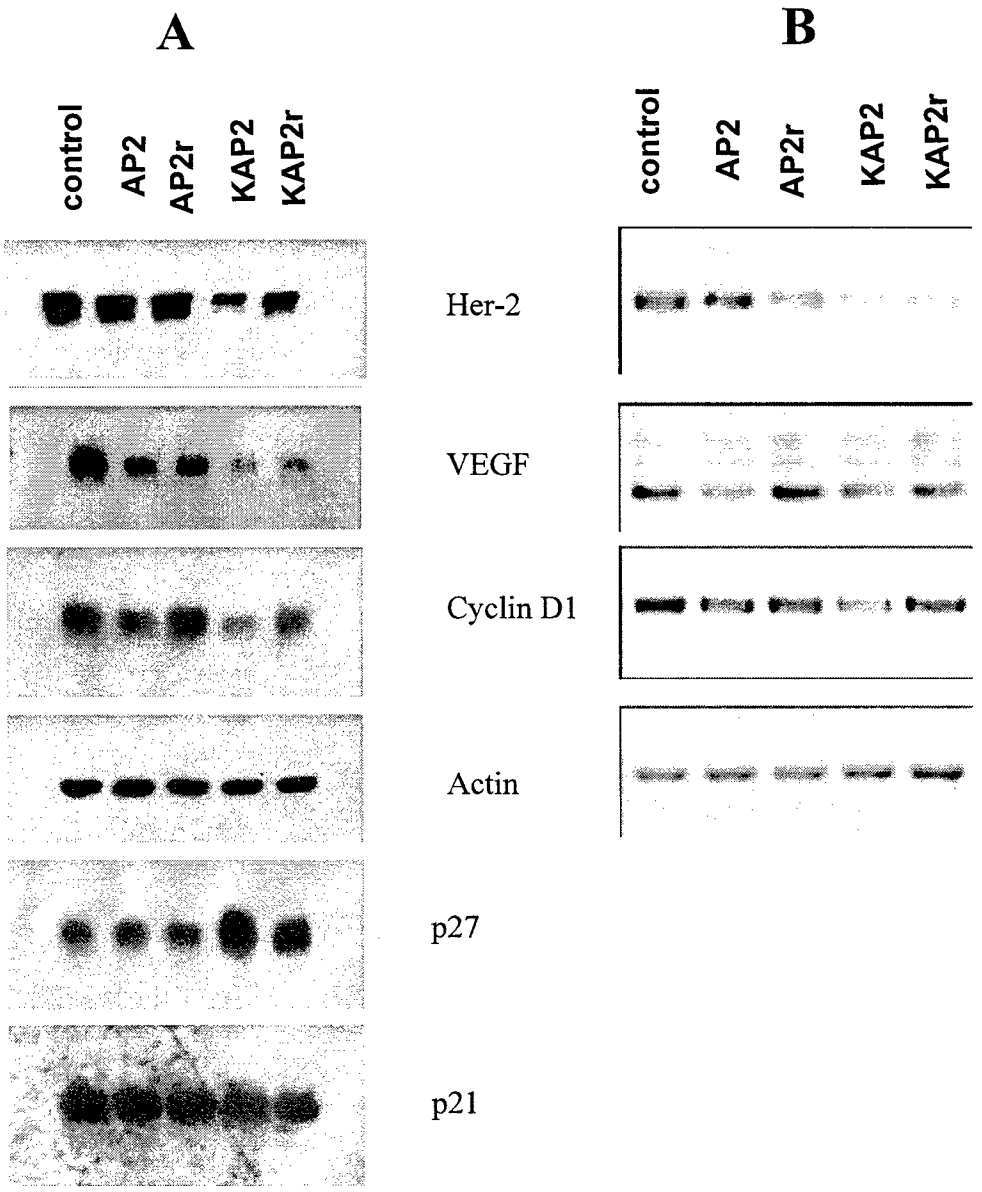
SkBr-3 cells were transfected with the plasmids as shown in the X-axis and incubated for 40 h. z-VAD-FMK (10μM) and TRAIL (50ng/ml) was added to the cells and incubated for 8 h (total incubation of 48 h). The cells were lysed and analyzed for caspase 3/7 activity. The data shows that KAP2 and KAP2r increase the caspase 3/7 activity indicating that they induce apoptosis.

Figure 15



Colony formation assay on SkBr-3 and MCF-7 cells shows that while AP-2 provides a growth advantage to the cells, KAP2 and KAP2r seem to provide a growth disadvantage. The SkBr-3 cells are more sensitive to the expression of KAP2 and KAP2r than the MCF-7 cells.

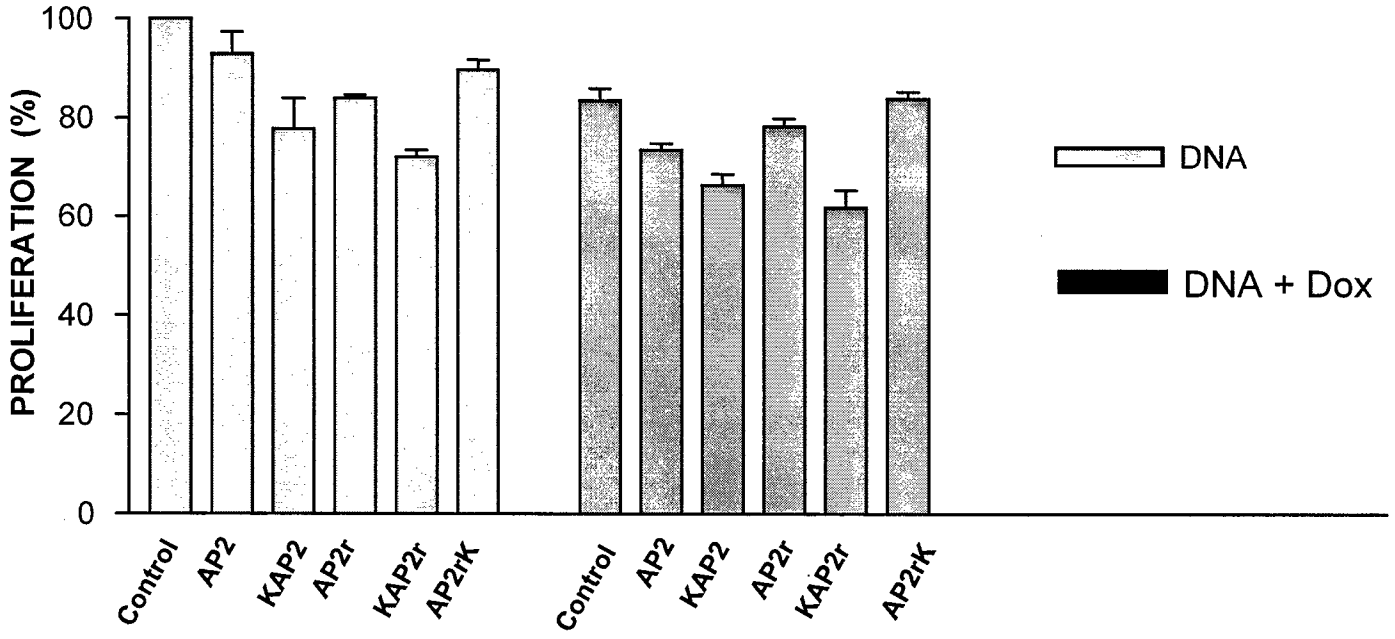
Figure 16



Western analysis (**A**) of the FACS sorted SkBr-3 cells shows that KAP2 and KAP2r reduce the protein levels of Her-2, VEGF and Cyclin D1, while increasing the levels of p27.
To verify if this effect was due to transcriptional repression, a semi-quantitative RT- PCR.analysis was carried out (**B**). The data shows that the cells expressing KAP2 and KAP2r indeed have lower levels of transcribed mRNA for Her-2, VEGF and cyclin D1. Currently, RT-PCR studies are being carried out to analyze the effect on p27, p21 and other proteins involved in cancer cell survival and apoptosis.

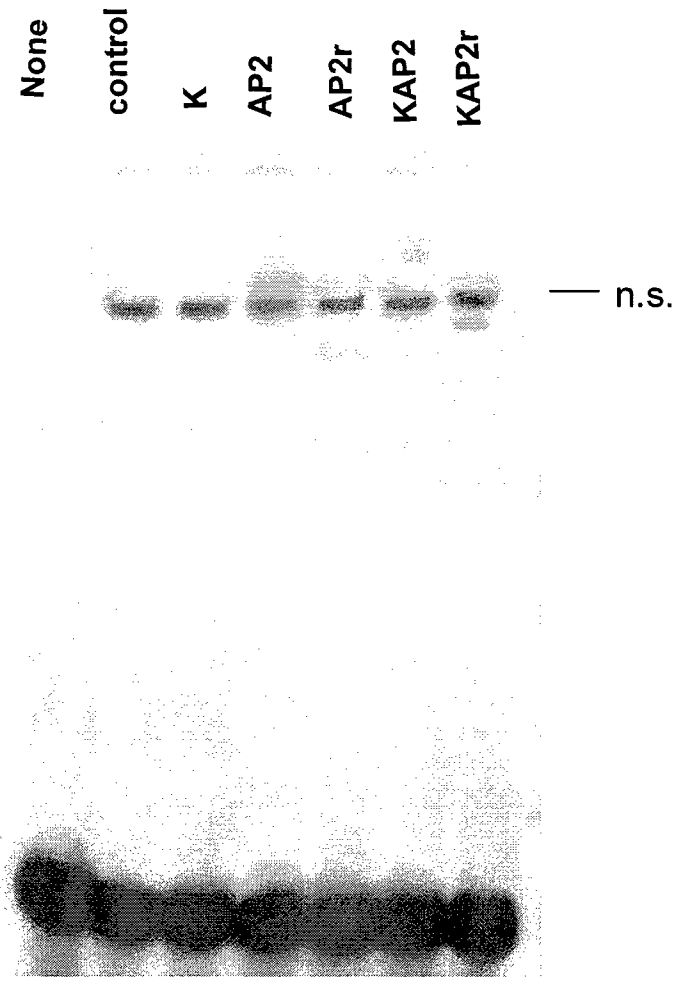
Figure 17

WST-1 ASSAY ON SKBR-3 CELLS



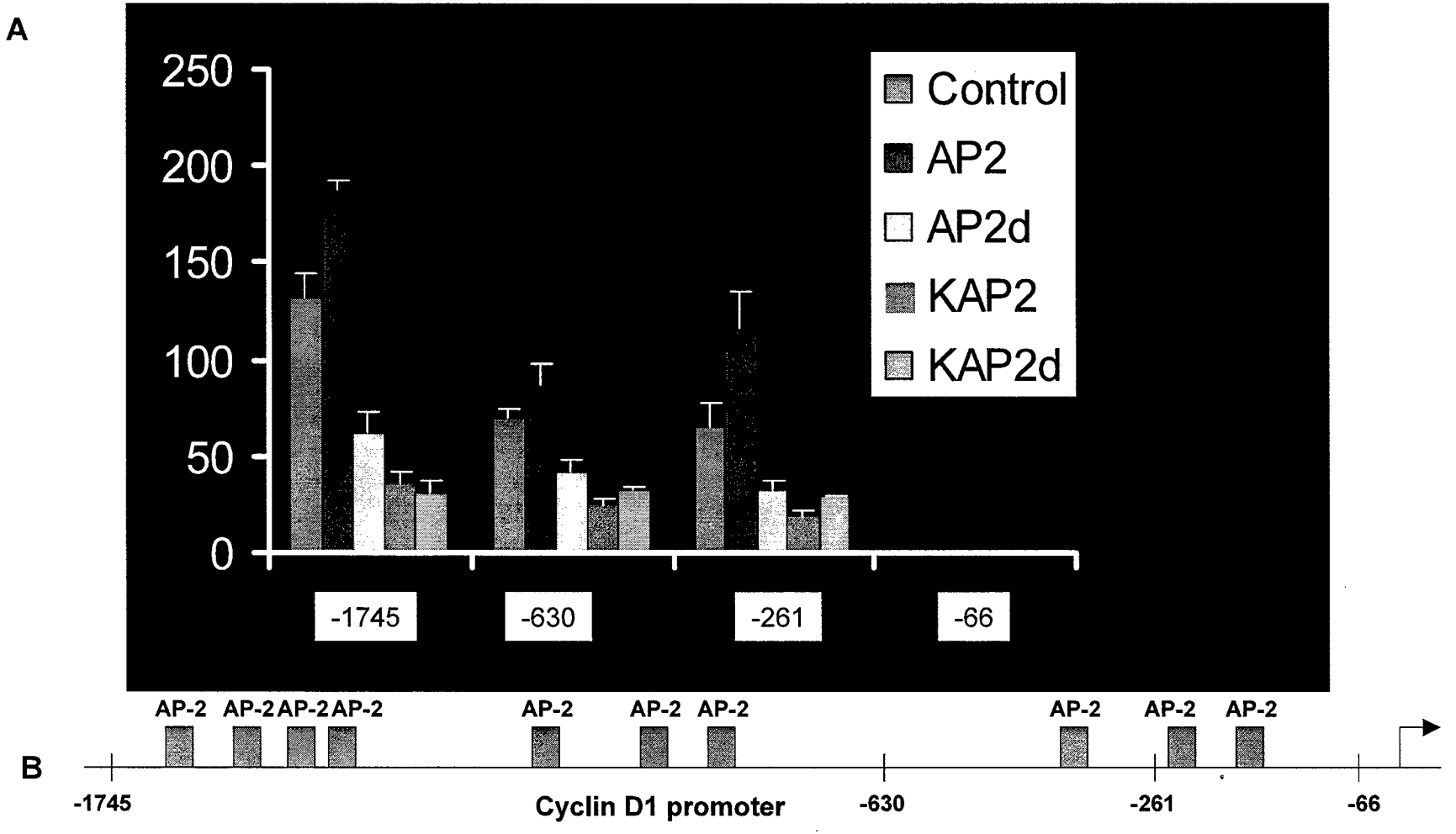
The WST-1 proliferation assay was carried out on transiently transfected SkBr-3 cells as described in the text. The data shows that the KRAB-AP2 constructs induce potent inhibition of cellular proliferation. Note that only 15-20% cell are transfected and the data shown is representative of 100% cells. Doxorubicin at 100nM concentration shows an additive effect rather than a synergistic effect with the KRAB-recombinants. This suggests that though co-treatment with doxorubicin induces an increased anti-proliferative effect, the effect is only additive, and may produce limited therapeutic benefit.

Figure 18



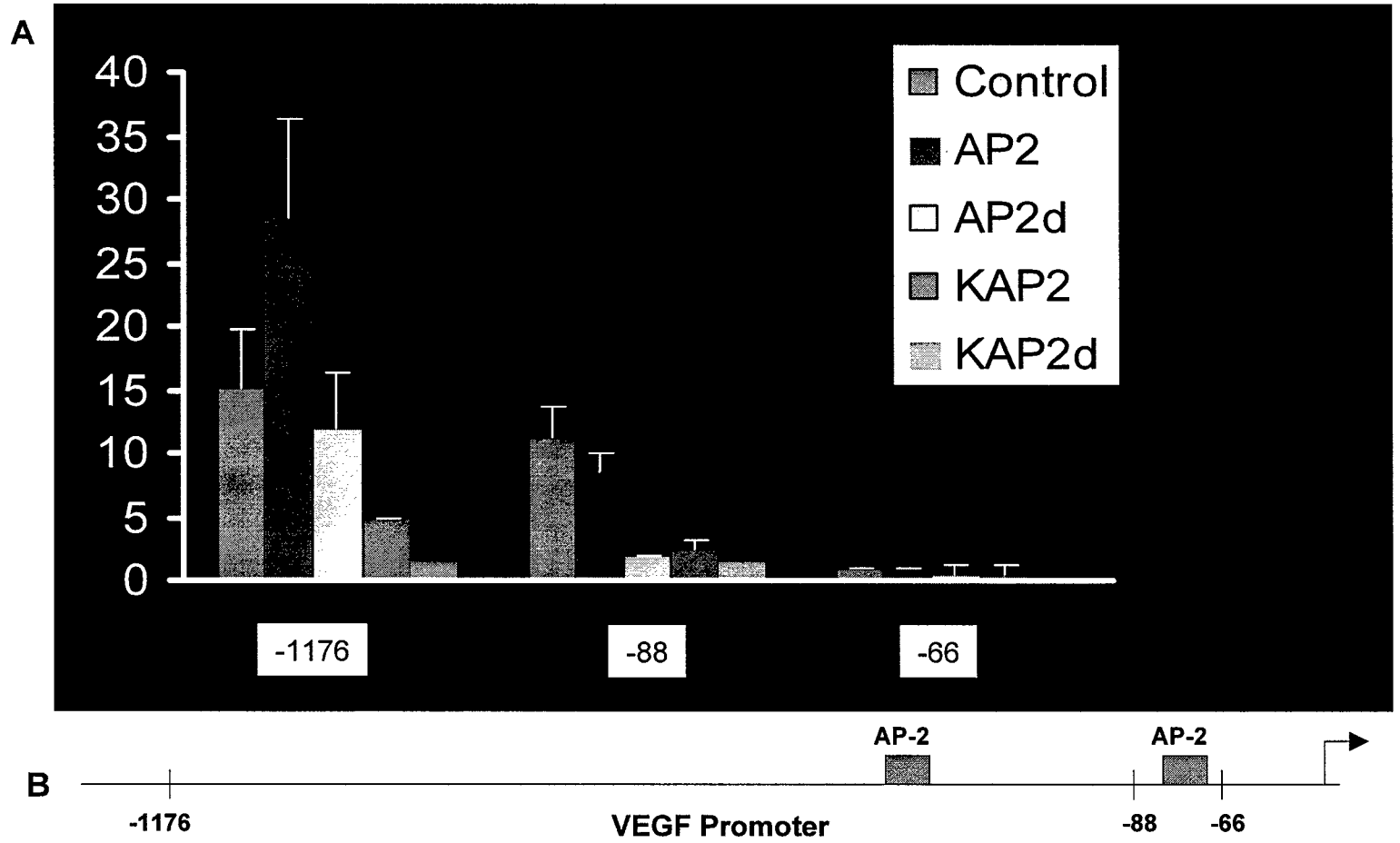
Nuclear extracts from transiently transfected HepG2 cells were incubated with a ^{32}p-labeled AP-2 consensus oligo and were resolved on a 6% polyacrylamide gel. The data shows that the lysates from untransfected and the KRAB domain transfected cells do not induce any mobility shift, except for a non-specific band (n.s.). The lysate from the AP2 transfected cells induces a shift, slightly higher than the non-specific shift. This was in accordance with previously published reports. Lysates from the AP2r, KAP2 an KAP2r transfected cells induced mobility shifts, that were consistent with their molecular weights and in comparison to the AP-2 shift, suggesting that these constructs retain their ability to bind AP-2 consensus DNA.

Figure 19



(**A**) AP-2 or the KRAB-AP2 plasmids were co-transfected in SkBr-3 cells with either of the cyclin D1 reporters shown in the schematic in figure (**B**). The data shows that AP-2 activates the cyclin D1 reporter, while the DNA binding domain and the KRAB-recombinants repress its activity. (**B**) The schematic shows the probable AP-2 sites on the cyclin D1 promoter as determined by the TRANSFAC Algorithm for finding transcription factor binding sites on gene segments.

Figure 20



(**A**) AP-2 or the KRAB-AP2 plasmids were co-transfected in SkBr-3 cells with either of the three VEGF reporters shown in the schematic in figure (**B**). The data shows that AP-2 activates the full length reporter, while the DNA binding domain and the KRAB-recombinants repress its activity. (**B**) The schematic shows the probable AP-2 sites on the VEGF promoter as determined by the TRANSFAC Algorithm and also from previously published reports on the VEGF transcriptional regulation.